THE IMMUNE RESPONSE IN HORSES TO VACCINATION AGAINST EQUINE INFLUENZA VIRUS. A COMPARISON OF RECOMBINANT DNA, INACTIVATED-VIRUS, AND MODIFIED-LIVE VIRUS VACCINES

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIRMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2001

ACKOWLEDGMENTS

Portions of this work were supported by an ongoing project within our laboratory funded by Hoechst Roussel Vet. I would like to thank Norbert Kline and his colleagues. I would also like to thank Mark Abdy for his help with the horse work and introducing me to Paul Gibbs. I would like to give credit to members of my committee, Bradley Bender, Jorge Hernandez, Calvin Johnson, Paul Lunn, and Parker Small for their guidance and suggestions through the development of this project. Individuals that were not on my committee but were no less helpful include, Dorothy Guantlett, Carlos Romeros, Steeve Giguere, Cynda Crawford, and Alysia Posey. I would like to give special thanks to my committee chair, Paul Gibbs for his wisdom and support in the development and completion of this project.

I was truly fortunate to have the support and prayer from friends and family. Thanks to Andy Albertson and Rick Mercer for a constant supply of encouraging words. Thanks to my parents Boyce and Marceil Sweat for their thoughts and prayers.

Possibly the greatest motivating force was the love and support from my wife Susan and daughter Jessica. I thank them for their sacrifices and patience throughout my absence.

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KEY TO ABBREVIATIONS

ANOVA	Analysis of variance
APC	Antigen presenting cells
C	Centigrade
CM	Culture medium
CMI	Cell-mediated immunity
Con A	Concanavalin A
CS	Clinical score
CTL	Cytotoxic T-lymphocyte
DMSO	Dimethyl sulfoxide
DTT	Dithiotreatole
EHV-4	Equine herpes virus-4
EID	Egg infectious dose
EIV	Equine influenza virus
ELISA	Enzyme-linked immunosorbant assay
ER	Endoplasmic reticulum
G3PDH	Glyceraldehyde-3 phosphate dehydrogenase
gE	Glycoprotein E
gI	Glycoprotein I
HA	Hemagglutinin
HAU	Hemagglutinating unit
HCL	Hydrochloric acid
HI	Hemagglutination inhibition
hrIL-2	Human recombinant interleukin-2
IACUC	Institutional Animal Care and Use Committee
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	immunoglobulin M
IL-4	Interleukin-4
IL-6	Interleukin-6
IM	intramuscular
IN	Intranasal
INF-γ	Interferon gamma
ISCOMS	Immune stimulating complexes
IURD	Infectious upper respiratory tract disease
M1&2	Matrix protein
MALT	Mucosal-surface-associated lymphoid tissue
MDCK	Madin Darby canine kidney
MHC	Major histocompatibility complex
MLV	Modified-live virus
MMLV	Moloney murine leukemia virus
NA	Neuraminidase
NALT	Nasal-associated lymphoid tissue

NP	Nucleocapsid protein
NS	Nonstructural
NVSL	National Veterinary Services Laboratory
OD	Optical density
OPD	O-phenylenediamine dihydrochloride
PBMC	Peripheral blood mononuclear cells
PHA	Phytohemagglutinin
RBC	red blood cell
Recomb	Recombinant
RNP	Ribonucleoprotein
rtPCR	Reverse transcription polymerase chain reaction
SC	Secretory component
SI	Stimulation index
SRH	Single radial hemolysis
TCID	Tissue culture infectious dose
TCR	T-cell receptor
TGN	*trans* Golgi network
Th1 and 2	T-helper
TNF-α	Tissue necrosis factor-alpha
UK	United Kingdom
URD	Upper respiratory tract disease
UV	Ultraviolet
VTM	Virus transport medium
WHO	World Health Organization

Abstract of Dissertation Presented to the Graduate School
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August, 2001

Chair: Dr. Paul Gibbs
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Equine influenza virus is a cause of upper respiratory disease that has economic significance to the horse industry. It is a highly contagious virus that is spread among horses despite vaccination. Due to antigenic changes in surface proteins and the ineffectiveness of vaccines, equine influenza has been maintained as an endemic disease within the equine population.

Various studies have shown that the use of inactivated-virus vaccines results in short-lived immunity that does not prevent infection but reduces the incidence of severe clinical disease. The implications of this partial immunity in equine influenza are two-fold. A sub-clinical infection will often not only show a reduction in performance, but serve as a potential threat to susceptible horses during racemeets and show events.

Because conventional inactivated vaccines against equine influenza are ineffective, novel approaches using recombinant DNA technology have been investigated. This study examines the

potency of a recombinant DNA vaccine consisting of an equine herpes-4 virus vector expressing the hemagglutinin and neuraminidase genes of the A/equine/2/Kentucky/94 strain of equine influenza virus. The immune response in horses was determined following inoculation with a recombinant DNA vaccine and compared to that seen after using a modified-live and inactivated-virus vaccine or natural infection. Horses were then challenged with equine influenza virus and the ability of each vaccine to prevent infection or reduce severe clinical disease was determined.

The results of this study indicate that the recombinant DNA vaccine examined here failed to induce an immune response due to over-attenuation. The modified-live and inactivated virus vaccines provided a reduction in the severity of clinical disease compared to that of an un-vaccinated control. Protection from severe disease was associated with serum antibodies in the inactivated vaccine recipients and serum, local, and cell-mediated immunity in the modified-live vaccinates. Horses initially infected with live virus were completely protected against infection upon challenge 8 weeks later. This study supports previous investigations and concludes that 1) inactivated-virus vaccines provide incomplete protection through humoral immunity, 2) modified-live vaccines induce serum and local antibody formation, and a cell-mediated immune response, and 3) repeat vaccinations are required to maintain immunity against infection with equine influenza virus.

CHAPTER I
LITERATURE REVIEW

Introduction

Equine influenza is an infectious upper respiratory disease of horses that has economic significance. Equine influenza has been the cause of outbreaks that have had an effect on race-meets, show and performance events, and breeding programs on an international level. Because of this, vaccines against equine influenza virus (EIV) infection have been investigated for the past 40 years. In 1956, EIV was first isolated in Prague, Czechoslovakia, and recognized as being a cause of upper respiratory disease in horses (Sovinaova, Tumova, and Pouska 1958). In 1963, a new strain was reported from horses at a racetrack in Florida and designated A/equine/2/Miami/63 (H3N8) (Wadell, Teigland, and Sigel 1963). Since the appearance of the type-2 strain of EIV, a number of sub-types have emerged leading to increased monitoring efforts and new strategies in vaccine development.

Due to the inability of conventional inactivated-virus vaccines to provide complete protection against infection and disease, novel vaccines such as recombinant DNA and modified-live preparations have been investigated. Studies including adjuvant-conjugated inactivated-virus (Hannant, Easeman et al., 1999;Mumford, Jessett et al., 1994a;Mumford, Wilson et al., 1994), modified-live (Holland, Chambers et al., 1999); (Lunn, Hussey et al., 2001), and DNA vaccines (Lunn, Soboll et al., 1999) have been conducted to determine the level of immunity and protection induced by each. Historically, the primary surrogate of immunity to EIV was an increase in antibodies to the hemagglutinin protein (Morley, Hanson et al., 1995). More recent studies have included cell-mediated effector mechanisms (Hannant, 1994;Hannant & Mumford,

1989;Hannant, 1994;Morley, Hanson, Bogdan, Townsend, Appleton, & Haines, 1995;Mumford & Wood, 1992). As the availability of equine-specific reagents increased, so did studies that characterize sub-isotype-specific antibody formation (Lunn, Olsen et al., 1999;Lunn, Hussey, Sebing, Rushlow, Radecki, Whitaker-Dowling, Youngner, Chambers, Holland, Jr., & Horohov, 2001;Nelson, Schram et al., 1998) and cytokine expression in horses (Giguere & Prescott, 1999). Investigations that examine a comprehensive immune response following vaccination or infection are still warranted, however. In this study, the immunological response of horses to vaccination against or infection with equine influenza was measured. To accomplish this, traditional and novel assay methods to quantify the immune response were optimized. Furthermore, the optimized methods were then used during animal studies that included immunization of horses with recombinant DNA, inactivated-virus, or modified-live vaccines.

Equine Influenza Virus

Equine influenza virus is one of a group of enveloped viruses in the family of Orthomyxoviridae which in the Greek translation means, ortho, "standard or correct," and myxo, "mucous." The name "influenza" can be traced to a Latin origin "influentia," or influence, because it was once believed that the cause of this disease had astrological or occult influences (Murphy, Gibbs et al., 1999). The current nomenclature was determined by the International Committee on Taxonomy of Viruses (ICTV) in the 1960s. To reference a particular strain, the system includes host origin, geographical location of the first isolate, strain number, and year it was isolated (WHO Memorandum, 1980). Orthomyxoviruses have a segmented, single stranded RNA, negative-strand genome. Because they are negative strand, they must not only contain a template for mRNA synthesis but also for anti-genomic (+) strand RNA (Fields, 1996). Orthomyxo viruses are similar to another group of viruses, the Paramyxoviridae, which have similar biological and structural properties. Influenza viruses use a number of unique and

complex mechanisms for the replication of viral proteins. Viral replication includes not only host protein-synthesis-machinery but host-cell RNA for primers as well. This mode of replication is a unique example of RNA that is not transcribed from DNA by RNA polymerase II.

Equine influenza virus is a type A virus. Influenza viruses, types A, B, and C, infect humans, but are of little importance in veterinary medicine and none have been recorded to cause clinical infection in the horse. The various types and strains of influenza viruses are separated from each other based on antigenic specificity resulting from structural differences. Some other unique properties of the influenza viruses are listed in the table below:

TABLE 1-1. Major Characteristics of Influenza Viruses

Type	Unique Properties
A viruses	Infect mammals including humans, swine and horses Infect avian species Surface proteins have a greater diversity as compared to B and C Contain eight distinct RNA segments (As does B)
B	Infect only humans Less complex surface glycoprotein amino acid sequence Contain eight distinct RNA segments (as does A)
C	Isolated from humans primarily but are also seen in swine Have a single multifunctional glycoprotein Contain 7 RNA segments

Adapted From Fields (1996)

Structural Design

The structure of the influenza virus varies slightly among types and has basic components made up of 0.08% to 1% RNA, 70% protein, 20% lipid, and 5% to 8% carbohydrate (Fields, 1996). Because influenza virus exits the infected cell through a process called 'budding", the

lipid envelope is essentially made up of the plasma membrane from the host cell. Influenza virus is morphologically recognizable resulting from the presence of the numerous (approximately 500, 10-14 nm) hemagglutinin (HA) spikes protruding from the lipid envelope (Laver & Valentine, 1969). Shorter in length, the mushroom shaped neuraminidase (NA) spikes are dispersed among the HA proteins. The ratio of these two membrane proteins is 5:1 HA to NA respectively (Zebedee & Lamb, 1988). Also present in the membrane is the viral matrix protein (M_1) and the transmembrane protein M_2 ion channel. Within the core of the viral structure are ribonucleoprotein (RNP) and nonstructural proteins (NS_1 and NS_2). The RNP structures consist of 8 separate segments with unique genomic products. Of particular importance in terms of antigenic recognition is segment 4 which encodes the HA protein, the major surface-glycoprotein sialic acid-binding structure. Other segments are responsible for the synthesis of P (polymerase) proteins (PB_1, PB_2, and PA), and nucleocapsid (NP) (the transcriptase complex) as well as NA, M_1, M_2 and NS_1 and $_2$ (Lamb & Choppin, 1976).

Structural Proteins and Genes

Polymerase proteins. PB2 and PA are responsible for encoding the largest of the viral proteins that are aggregated in the cytoplasm and nucleus of infected cells. The PB1 protein has a key role in catalyzing nucleotide polymerization and chain elongation (Braam, Ulmanen et al., 1983).

Nucleocapsid proteins. The NP reacts with other RNA segments to form a nucleocapsid. The NP protein has been the subject of research to determine homology between different strains. Cytotoxic T lymphocytes against influenza A virus will non-specifically cross-react with NP subtypes of influenza A virus isolated from humans and mice (Yewdell, Webster et al., 1979). The NP is synthesized in the cytoplasm, complete with specific residues that provide targeting to the nucleus for association with the various RNA strands for assembly (Lin & Lai, 1983).

Hemagglutinin proteins. The HA has a molecular weight of 61,468 with 1,778 nucleotides; it binds to the sialic acid glycoprotein receptors on erythrocytes and other cells, and is responsible

for attachment and penetration into the host cell (Fields, 1996). There is often considerable antigenic variation between the HA protein of different strains of influenza virus and thus the HA has a significant role in "defining" the susceptible host. The HA protein mediates fusion of the virus membrane and the endosomal membrane facilitating the release of the nucleocapsid contents into the cytoplasm of the host cell (Fields, 1996). Structurally, the HA starts as a homotrimer manufactured in the endoplasmic reticulum (ER) designated H_0 (Wiley & Skehel, 1977). After modifications of amino acid residues added to the carboxy-terminal, the HA molecule is targeted to the ER membrane by an N-terminal signal peptide. The integral membrane protein then consists of two segments with a di-sulfide linked region (Fields, 1996). The H_1 segment consists of 319 to 326 residues and is cleaved leaving an H_2 segment (Verhoeyen, Fang et al., 1980). The cleavage site can consist of up to 6 residues depending on the subtype and shows the loss of a single arginine residue. The loss of an arginine residue would indicate a trypsin-like or carboxypeptidase B type enzyme which is responsible for the activation of the HA protein during attachment (Dopheide & Ward, 1978). During infection, changes occur to the HA molecule within endosomes. HA molecules within the low pH environment of the endosome are cleaved, exposing a fusion peptide. Depending on the virus strain, the type of cells infected, and growth conditions, the HA can be whole (HA_0) or cleaved into HA_1 and HA_2. The HA protein must be cleaved into HA_1 and HA_2 for the virus to be infectious (Lamb, 1989). The fusion peptide requires the cleavage of HA_0 into HA_1 and HA_2 for activation. This activity has a specific temporal sequence which must be exact to avoid the formation of oligomer aggregates. Cleavage of the connecting peptide of the HA molecule occurs in response to different compounds. Furin is an intracellular enzyme found within the *trans* Golgi network which will cleave HA (Stieneke-Grober, Vey et al., 1992). Hemagglutinin from virus grown in cell-culture systems requires a source of exogenous trypsin if the HA contains an arginine residue in the connecting peptide; this is seen in all human strains. When influenza is cultured in embryonated eggs, a different enzyme proposed to be a factor Xa-like protease is

responsible for cleavage of the HA (Kawaoka & Webster, 1988). Virulence versus non-virulence is thought to be related to the presence of furin recognition motifs or a single arginine residue respectively (Nestorowicz, Kawaoka et al., 1987).

As previously mentioned, the HA is a major component of antigenic recognition by neutralizing antibodies. The epitopes of the HA are made of two regions; one extends approximately half way up the trimer (HA_2 region) and a second at the globular head of the molecule (HA_1). These epitopes form the HA receptor binding site at the end of each HA_1 and HA_2 sub-unit (Gibson, Daniels et al., 1988). Antigenic variations are demonstrated by changes or substitutions at specific amino acid residue sites ((Rogers & Paulson, 1983). Antigenic relatedness has been established by comparing the homology between different segments of the HA protein. By comparison, nucleotide and amino acid substitutions are implicated in low levels of antigenic drift. For example, 16 subtypes of type 1 equine influenza isolated during a period ranging from 1956 to 1977 were compared based on amino acids deduced from nucleotide sequences of viral RNA for the HA protein. Additionally, the genomic sequence of the HA from two other influenza viruses from avian origin (A/FVP/Rostock/34 (H7N7) (Porter, Barber et al., 1979) and seal origin (A/seal/Massechusetts/1/80 (H7N7) were compared (Webster, Hinshaw et al., 1981). When the HA gene nucleotide sequence of A/equine/Prague/53 subtype was compared to the Rostock avian and Massachusetts seal subtypes, the signal peptide, the HA1, and the connecting peptide, consisting of 1,068 bases, showed homologies ranging from 55 to 77 percent. When amino acid extensions at the carboxy-terminus were compared among this group, an even higher percentage of homology was noted ranging from 79% to 84%. This study showed that a high degree of homology was present between strains that were considered to share an ancestral relationship. In contrast, another study comparing avian and equine species of influenza revealed no carboxy-terminus extensions in the avian subtypes which are characteristic in equine influenza (Fields, 1996). A similar study comparing equine subtypes Prague/56 and Cambridge/63 revealed a high degree of similarity with only 6 nucleotide differences in the HA1

coding sequence (Webster, 1993). Five such nucleotide changes resulted in amino acid substitutions. Of particular interest was the change of Thr to Ile at site 160 in the Cambridge/63 virus; a potential glycosylation site. Additions and deletions of amino acids at these potential glycosylation sites are considered to be relevant when comparing the relatedness of strains but, more importantly, are thought to be associated with virulence. These areas are regarded as important functional (fusion properties) sites based on their three-dimensional structures (Daniels, Skehel et al., 1985).

Modifications and conservation in amino acid sequences are implicated in affecting the infectivity of an emerging subtype. When the A/Prague/63 HA1 sequence was aligned with a human influenza A (H3N2), cysteine residues were found to be important in maintaining the three-dimensional structure as well as other various residues (Daniels, Skehel, & Wiley, 1985;Rogers & Paulson, 1983). Additionally, all strains of EIV-1 have a conserved glutamine at site 226 which has significant binding specificity with the alpha-2:3 sialic acid receptor. This indicates a conserved function within the HA molecule of different strains.

The rate at which both silent mutations and amino acid substitutions occur is believed to be a result of the frequency of replication cycles and can have significance in strain diversity (Fields, 1996). The rate is highest in human influenza followed by swine and equine with the slowest being noted in avian strains (Bean, Schell et al., 1992). From rate comparisons, equine-1 A viruses are divided into two distinct groups from 1956-63 and 1964-77. The silent mutation rate of HA1 for the two periods was calculated to be 0.014%/year and 0.169%/year and the amino acid substitution rate is 0.214%/year and 0.412%/year respectively (Gibson, Daniels et al., 1992). This is consistent with other published data reporting that equine influenza viruses isolated before 1968 had substitution rates of 3.1 nucleotides and 0.8 amino acids per year whereas strains from 1968 and beyond accumulated 7.9 nucleotide and 3.4 amino acid substitutions per year (Fields, 1996).

Neuraminidase. Neuraminidase (NA) membrane protein is a homotetramer that has a molecular weight of 50,087, contains 1,413 nucleotides, and possesses both biological and antigenic significance(Laver, Colman et al., 1984). The NA is a class II integral membrane protein which consists of: 1) a trans-membrane stalk region with a high degree of variability between both A and B virus and sub-types in regards to both length and amino acid sequence; 2) a head region with varying degrees of homology among A and B and A subtypes; and 3) a cytoplasmic tail which is conserved among A but not between A and B viruses (Fields, 1996). Each monomer is made of 4-stranded anti-parallel beta-sheets which fold into six identical units. Each unit makes up a binding pocket (Colman, Varghese et al., 1983). Neuraminidase is believed to be important in initiating infection of cells of the respiratory system by facilitating transport of the virus through the mucin layer. Its biochemical activity includes the ability to bind and remove sialic acid by catalyzing the cleavage of alpha-ketosidic linkages between HA and NA and the host cell surface (Palese, Tobita et al., 1974). Some NA cause hemagglutination; this feature has been used to identify subtypes (Webster, Hinshaw, Bean, Van Wyke, Geraci, St Aubin, & Petursson, 1981).

M1 and M2. The viral matrix protein (M1) is the most abundant protein in the influenza virion and is believed to be a structural protein underlying the lipid envelope. The M1 interacts with the cytoplasmic tails of HA, NA, and M2 proteins as well as RNP structures (Rees & Dimmock, 1981). This was determined by a study that showed treatment with amantadine (1-aminoadamantane hydrochloride) inhibited the flow of ions into the virion during uncoating, thereby resulting in the dissociation of RNP from the M1 protein (Martin & Helenius, 1991).

M2 protein is a type III integral membrane protein homodimer with disulfide-linked pairs that form a tetramer (Holsinger, Nichani et al., 1994). Several copies are synthesized during replication with 20 to 60 molecules being integrated into the whole virus. These are expressed during replication on the apical surface (site of virus budding) of the infected host cell. The M2 protein consists of N-terminal extracellular residues, a trans-membrane domain which is

responsible for forming the Na+ and hydronium permeable ion channel, and a cytoplasmic tail (Pinto, Holsinger et al., 1992) The M2 molecule is an important target of anti-viral therapy with amantadine (1-aminoadamantane hydrochloride) which disrupts ion transport (Oxford & Galbraith, 1980).

Viral Transmission

The influenza virus particle makes multiple low-affinity attachments to sialic acid-containing receptors through galactose, alpha 2,3 or 2,6 linkages (Weis, Brown et al., 1988). The appropriate host cell, containing clathrin-coated sections of plasma membrane, engulfs the virus particle through a process called receptor-mediated endocytosis, formerly referred to as viropexis. Upon entry into the cell, the coated membrane-bound vesicle releases the virus by fusion into endosomes. The endosomes provide an environment of decreasing pH necessary for the processing of HA which leads to fusion of the viral envelope with the endosome membrane. In some human strains, this process takes about 20 to 35 minutes and is required before the matrix protein can release the RNP for transport into the nucleus via nuclear pores. However, before the M1 protein can dissociate from the RNP, a requisite flow of ions from the endosome compartment through the M2 ion channel into the virus core occurs. It is also postulated that the flow of ions into the virus further "prepares" the transmembrane portion of the HA molecule for fusion earlier on in this process. This is the point where antiviral drugs are targeted by utilizing the fact that acidotropic weak bases such as amantadine and chloroquine will raise the pH within endosomes (Matlin, Reggio et al., 1982). The idea of low pH leading to uncoating is consistent with the more neutral pH at the cytoplasmic membrane, promoting virion assembly. Low pH is proposed to facilitate the dissociation of the cytoplasmic tail of the HA protein from M_1 protein and intact RNP (Wharton, Belshe et al., 1994). This is followed by entry into the nucleus and subsequent mRNA transcription of RNP.

RNA Replication and Protein Synthesis

The influenza virus is a single-stranded RNA virus equipped with some, but not all of the proteins needed for replication. Within infected cells, viral RNA is transcribed into mRNA and replicated. Synthesis of viral mRNA requires a host-cell RNA polymerase II as well as host RNA as a source for primer sequences which are generated by viral endonucleases (Fields, 1996). A cap-dependent endonuclease will cleave sections from a methylated 5'-capped cell RNA 10 to 13 nucleotides from the end, preferentially at a purine residue (Panniers & Henshaw, 1983). In the presence of viral RNA associated with NP, PG1, PB and PA, transcription can proceed with a G residue incorporated at the 3' end (Inglis, Carroll et al., 1976). Specifically, PB1 adjacent to the G residue is reported to catalyze the addition of individual nucleotides. PB2 is responsible for recognizing and binding to the cap primer sequence. Messenger RNA is synthesized until the termination sequence of 5 to 7 uridine residues is reached (Rogers & Paulson, 1983). A poly-A tail is then added to the 3' end of the nascent chain (Inglis, Carroll, Lamb, & Mahy, 1976). Viral RNA replication not only includes the production of a template strand, but its subsequent copying back into viral RNA.

It appears that primers derived from host-cell RNA polymerase II transcripts are required, which explains the activity of actinomycin D and α-amanatin (Plotch, Bouloy et al., 1981). Viral RNA replication is completed in two events: 1) the synthesis of full-length copies of an RNA template; and 2) the subsequent copying of these templates into viral RNA. Influenza virus gene expression is based on quantitative changes in the transcription of specific RNA segments (Lamb & Choppin, 1976). Unlike DNA viruses, influenza virus gene expression occurs in distinct stages that couples the synthesis of viral mRNA, viral RNA, and viral proteins (Hay, Abraham et al., 1977). The selective copying of a particular template RNA into viral RNA results in the subsequent production of mRNA and its corresponding protein. For example, NS and NP viral RNA are synthesized early, whereas the M1 is produced at a later point during replication. These events occur within the nucleus where the products remain until the appropriate time for egress

and assembly is reached. The timing of these events is closely associated with a sequence of viral protein and RNA production. The "late phase" product, M protein, basically signals the end of mRNA production and thus results in the assembly and transport of viral components from the nuclease into the cytoplasm (Martin & Helenius, 1991).

Integral membrane proteins HA, NA, and M2 are synthesized in the cytoplasm on membrane bound ribosomes. Trafficking of the proteins from the ribosomes through the *trans* Golgi network (TGN) is via a signal recognition particle-dependent system. For HA proteins, a transitional sequence of appropriate folding steps occurs as they progress through the ER and Golgi (Boulay, Doms et al., 1988). At this point amantadine can cause a premature HA conformational folding resulting in an extrusion of the fusion peptide at an inappropriate time within the wrong sub-cellular compartment. This in turn inhibits the budding of an infective influenza particle. Other post-transcriptional changes are made to the membrane proteins before they exit the TGN. Complexed forms of HA and NA containing sialic acid and multi-basic residues on their carbohydrate chains are cleaved in the TGN by an endoprotease, furin (Stieneke-Grober, Vey, Angliker, Shaw, Thomas, Roberts, Klenk, & Garten, 1992). Later, integral membrane proteins followed by M_1-associated RNP are transported to the apical-surface of the cell membrane prior to budding (Hull, Gilmore et al., 1988). The release of a fully formed viral particle requires neuraminidase; defective NA results in large aggregates of virus particles (Palese, Tobita, Ueda, & Compans, 1974).

The Significance of Equine Influenza

The prevalence and incidence of equine respiratory disease has been well documented in association with equine influenza virus (EIV). Understanding the pathogenicity of a disease affecting a species that possesses such a significant recreational and economic value is of paramount importance. Although there were suspect cases centuries ago, the earliest documented case of an EIV outbreak in horses was in Prague, Czechoslovakia, in 1956; the virus isolated was

designated A/equine/Prague/1/56, (H7N7)(Sovinaova, Tumova, & Pouska, 1958). The next significant subtype of EIV to be isolated was A/equine/Miami/1/63 (H3N8) (Wadell, Teigland, & Sigel, 1963). These two strains of EIV are commonly referred to as equine 1 and equine 2 respectively and the type 2 virus is considered to be more virulent (Wood, Mumford et al., 1983) and possesses the potential to cause severe disease in young horses (Beech, 1991). While antibodies persist in unvaccinated horses, no infections by the subtype A/equine/1 have been reported since 1979. However, a recent screening of 359 serum samples collected from unvaccinated horses in Bosnia Herzegovina indicated that antibodies to A/equine/1/Prague/56 influenza virus were detected in 8.3% of the samples using the HI test (personal communication to Paul Gibbs from Ramiz Velic).

Based on changes in the HA and NA genes, A/equine/2 strains show a division into two subgroups. The newly characterized H3N8 virus underwent sufficient antigenic drift at the HA and NA level to result in two variant subtypes, one including the A/Miami/63 (prototype), A/Tokyo/71, and A/Switzerland/79 and a second represented by A/Fontainblue/79, A.Newmarket/79, A/Solvalla/79, and A/Kentucky/81 (prototype variant)(Wilson, 1993). Increased efforts in surveillance for the A/equine/2 virus has resulted in the recognition of geographic divergence into two further categories, the American-like (isolated from horses in USA and Argentina) and the Eurasian-like (isolated from horses in Europe, Mongolia and China) (Wilson, 1993).

Host Specificity

While there are examples of "host specific" influenza viruses in mammals such as those seen in humans, whales, pigs, and horses, they are believed to be related to strains of avian virus (Hinshaw, Bean et al., 1986;Webster, Hinshaw, Bean, Van Wyke, Geraci, St Aubin, & Petursson, 1981;Guo, Wang et al., 1992). Evidence for a conserved lineage between swine and human influenza strains is found in the fact that both have similar NP genes arising from a common

origin. Additionally, NP genes of an avian origin have been determined in mink, A/Mink/Sweden/84, swine A/Swine/Netherlands and A/Swine/Germany/81, and horses A/Equine/Jilin/89 (Fields, 1996). One study showed that the amino acid sequences of the HA1 molecules of A/duck/Ukraine/63 and A/equine/Miami/63 had similar "silent-mutation" rates. From this, Daniels et al. deduced that a common ancestral virus existed around 1945 (Daniels et al., 1985).

The pathogenicity of a particular strain is closely related to the HA protein. The attachment of virus to the host-cell is accomplished through interactions between the HA and sialic acid-containing glycoproteins, specific for an alpha 2:3 linkage. Furthermore, host specificity is a result of a glutamine residue in animal strains (seen in A/equine/Miami/63, A/equine/Fontaineblue/79, and A/duck/Ukraine/63) instead of a leucine seen in the A/ human X-31 virus/69 at site 226. This is an example of a point mutation that can have relevance to antigenic drift as discussed later.

Another possible explanation for the heterogeneity of the various strains of influenza is through evolutionary pressure exerted by the immune system of the host species, sometimes referred to as " host immune-selection pressure." This occurs most dramatically where the number of replications is high. Because humans provide a large reservoir of susceptible hosts, the number of replications is high, allowing the HA surface protein to evolve at a much quicker rate than other species (Bean, Schell, Katz, Kawaoka, Naeve, Gorman, & Webster, 1992). In comparison, nucleotide changes in influenza of avian origin occur much more slowly and do not result in amino acid changes. This silent mutation in avian viruses has resulted in a phylogenetic profile that apparently has not changed in over 50 years (Gorman, Bean et al., 1991). This concurs with the idea that conservation within the avian species is a result of these viruses reaching their adaptive potential where nucleotide changes afford no advantage for selectivity. This has significance relative to human infection; some of the viral gene segments that caused

pandemics in humans in 1918, 1956, and 1968 still persist within avian species with few or no mutations (Gorman, Bean, Kawaoka, Donatelli, Guo, & Webster, 1991).

Shift and Drift

Antigenic shift is the result of reassortment of genes between viruses of two strains. Reassortment occurs in viruses with segmented genomes and has a low rate of occurrence. It can result in the emergence of a highly virulent strain (Rott, Orlich et al., 1984). An example of this was the reassortment of genes between avian and human virus strains to produce the 1957 Asian "flu" outbreak. This new, antigenically distinct strain derived its HA, NA, and PB1 genes from an avian virus while the remainder of the genes were of human origin. (Kawaoka & Webster, 1989;Scholtissek, Koennecke et al., 1978). Other reassortments have occurred between other mammals and avian species (Guo, Wang, Kawaoka, Gorman, Ito, Saito, & Webster, 1992). Within a period of little more than a year, two new strains of EIV were characterized from the northeastern region of China (Guo, Wang, Kawaoka, Gorman, Ito, Saito, & Webster, 1992;Guo, Wang et al., 1995). Emerging strains of EIV, as in human influenza, can cause severe clinical disease based on the absence of serum antibodies that recognize antigenically similar strains. The two strains isolated in northeastern China were determined to be antigenically similar to each other and to a H3N8 subtype A/equine/2/Miami/1/63 strain and were referred to as Jilin/1/89 and Heilongjaiang/15/90. Phylogenetic analysis of complete and partial sequences of the HA, NP, M, and NS genes revealed that Jilin/1/89 was most similar to an avian H3 virus, A/Duck/Ukraine/3/63. While the reassortment of genes can cause pandemic viruses to emerge, another possibility is that a virus that has been out of circulation for a number of years can emerge again due to the birth of a new susceptible population in the intervening period. This was speculated as being the cause of the 1977 pandemic of human influenza in China due to a Russian strain which had not been seen for 27 years (Nakajima, Desselberger et al., 1978).

RNA viruses, which include EIV, have the capacity to undergo spontaneous mutations (point mutations) which result in phenotypic changes (Fields, 1996). This is called antigenic drift. In one study, 79% of changes in the amino acid sequence between two equine strains (Miami/63 and Fontainebleau/79) were in the HA1 gene, which highlights the importance of the antigenicity of HA1 (Daniels, Skehel, & Wiley, 1985). The rate of changes that occurs is highest in humans, followed by equine and swine, with the lowest being found in the highly conserved avian strains. In a study conducted by Skehel and others, the rate of amino acid substitutions in avian species was 0.36%/year over a 16-yr period as compared to human influenza which is about 0.87%/year over 14 yr (Skehel, Daniels et al., 1983). Silent mutations consisting of nucleotide changes that do not result in amino acid substitutions in the HA1 coding region, follow a similar trend (0.17% for horses and 0.3% for humans). The rate of changes is presumed to be a function of the number of replication cycles and not due to species-specific factors. There are five antigenic sites on the HA1 protein A to E which are most susceptible to changes resulting from point mutations. The nucleotide changes in these distal regions of the molecule HA1 are the basis for virus evasion from antibodies generated in the host by previous exposures (antigenic drift). An interesting point is that immunologically induced pressure is believed to affect the selection of mutants in human and other mammalian species whereas in avian species, this is less the case (Air, Gibbs et al., 1990). This is due to the fact that avian viruses have an evolution rate (nucleotide change rate) that does not result in amino acid changes. Because the nucleotide rate is slower and does not lead to amino acid changes, it appears that avian influenza virus has reached its optimum capacity to adapt.

Mode of Transmission and Infection

The natural route of infection for horses is through direct transmission by aerosol or indirectly through fomites (Ames, 1988). Aerosol transmission is the more important route. Experimental infection protocols have included intranasal inoculation and aerosolized mist with

nebulizers as a means of administering live influenza virus (Mumford, Wood et al., 1983;Mumford, 1991;Mumford, Wilson, Hannant, & Jessett, 1994). In humans, natural and experimental infections are through person-to-person contact or via the aerosol route respectively (Douglas, 1975). The number of infectious particles required to produce clinical signs in horses has been investigated by Mumford and others with experimental doses ranging from 10^6 EID_{50}/mL using a nebulizer and $10^{8.7}$ EID_{50}/mL dose during intranasal instillation (Mumford, Wood, Scott, Folkers, & Schild, 1983;Hannant, Jessett et al., 1989). During natural infection, it is speculated that fewer virus particles are needed to disseminate the disease. It is rare to isolate a titer of more than 10^3 EID_{50}/mL from horses naturally infected with EIV (Mumford, Wilson, Hannant, & Jessett, 1994). The average size of a particle generated by humans during a sneeze or cough is less than 2 micrometers in diameter and could contain 1 $TCID_{50,}$ most likely deposited in the lower respiratory airways of the lung (Knight, 1973). The explosive cough from a horse with EIV can result in the effective aerosolization of viral particles. No studies have been conducted to determine the size of aerosolized particles generated from horses or the amount of virus contained in each. However, what has been well established is that the target cells of EIV are in the upper and potentially lower respiratory tract (Beech, 1991).

Clinical Presentation

Under optimum conditions of transmission, where infected (possibly sub-clinically) horses co-mingle with those that are susceptible, the onset of influenza can be acute and the dissemination rapid (Ames, 1988). The virus is shed during the incubation period and horses can remain infectious for up to 5 days after the appearance of clinical signs (Murphy, Gibbs, Horzinek, & Studdert, 1999). Elevated temperatures from 39.1 to 43.3 ^{0} C can last from 2 to 5 days accompanied by a serous-type nasal discharge. Often this precedes a secondary bacterial infection with an associated mucopurulent discharge (Ames, 1988). In some cases, mortality can be a result of bacterial as well as viral pneumonia (Baker, 1986). In a 1994 epidemic in China,

30,000 horses died and 1.5 million became seriously ill when bacterial infections complicated an EIV outbreak. (Shortridge & Watkins, 1995). An unproductive, yet explosive, cough is harsh and can persist for up to 3 weeks. Anorexia, depression, tachypnea, tachycardia, and myalgia can also be associated with clinical disease related to EIV (Coggin, 1979). Reports of cardiomyopathy have been noted in rare cases. In humans, myositis with an increase in serum muscle enzymes (myoglobinemia and myoglobinuria) and accompanied by pain and swelling of the limbs can occur (Middleton, Alexander et al., 1970). Similar signs have been documented in horses; they appear stiff and are hesitant to move with a slight swelling in the limbs (Beech, 1991). During a recent study, horses which where seronegative to EIV (A/Eq/Prague/56, A/Eq/Miami/63, A/Eq/Saskatoon/90, and A/Eq/Kentucky/91) were challenged with an aerosol of A/Eq/Kentucky/91 at $10^{8.68}$ EID_{50}/animal (Morley, Gross et al., 1998). After experimental-infection, some horses were exposed to training on a treadmill and some were rested in their stalls. Horses exercised during the influenza infection showed an increase in the severity of clinical signs. Specifically, fever, coughing, mucopurulent nasal discharge, anorexia, and lethargy persisted longer and were more severe in the exercised horses infected with EIV than those which were rested. Additionally, exercised horses lost more body weight than did their cohorts. Another study showed that permanent damage to the myocardia can occur through interstitial myocarditis present in horses after infection with A/Equine/Miami/1/63 (Gerber, 1970).

Pathogenesis

The pathogenesis of influenza has been described for humans (Small, 1990) and horses, (Beech, 1991;Coggin, 1979). Virus particles pass through the mucous layer and adhere to ciliated columnar epithelial cells. These cells appear vacuolated, edematous, and lose cilia before they are desquamated (Beech, 1991). Damage to the respiratory epithelium results in a decrease of respiratory clearance leading to secondary bacterial infection in humans (Small, 1990). Similar to

humans, horses are susceptible to secondary respiratory-tract infections (Beech, 1991). Around 1 day post-infection, the number of mucous producing and ciliated cells will be reduced to the point where areas of thickened and hyalinized basement membrane are exposed. Sub-mucosal edema and hyperemia results from infiltrating inflammatory cells, neutrophils and mononuclear cells (Coggin, 1979). Additionally, alveolar macrophage function is reduced (Ames, 1988). At 3 to 5 days after the onset of illness in humans, regeneration of the epithelium is initiated by the differentiation of basal cells into serous and ciliated cells (Small, 1990). What is both interesting and biologically practical is the fact that basal cells are not susceptible to infection by influenza virus. If this were indeed the case, infection in humans and animals with the influenza virus would be fatal to the host and self-limiting. The apparent reason for the inability of basal cells to become infected is due to their lack of the proper receptors for influenza (Small, 1990).

Epidemiology

Outbreaks can involve horses of varying ages and immunity to EIV but are most commonly seen among animals from 1 to 3 years of age (Ames, 1988;Livesay, O'Neill et al., 1993). An outbreak of upper respiratory disease (URD) in standardbred horses in Canada over the 2-yr period of 1973 to 1974 showed an age-specific infection associated with EIV. In the first and second years of the outbreak, 2 yr-old horses had the highest attack rate among a group of 2 to 9 year-olds at 0.24 and 0.25 for each year respectively (Sherman, Mitchell et al., 1979). As would be expected, the severity of the infection was reported to be dependent upon prior immunity to EIV. Horses with pre-existing serum antibody titers to EIV will have less severe clinical signs and will shed less virus for a shorter period of time (Hannant, Mumford et al., 1988). It seems reasonable that outbreaks have a better chance to occur when sub-clinically infected horses are mixed with naïve young horses not previously exposed to or vaccinated against EIV. This situation often presents itself at race-tracks, shows, and breeding farms. In the previous example, Sherman et al., 1979 described meets held in Ontario, Canada during winter,

spring, and fall where standardbred horses had episodes of URD associated with equine influenza and rhinopneumonitis virus. As reported with the human strains of influenza (Fields, 1996), EIV survives in aerosol in a relatively low humidity, low temperature environment which can be seen in stables at holding facilities or during the winter months (Beech, 1991).

Despite the existence of vaccines over the last 30 years (Bryans, Doll et al., 1966), EIV has circulated within equine populations in North America, Europe, and Scandinavian (Powell, Watkins et al., 1999). Recent epidemics (i.e. last 20 years) have been reported in the United Kingdom, China, India (Mumford, 1999a), Hong Kong (Powell, Watkins et al., 1995) and Croatia in 1993 (Madic, Martinovic et al., 1996). When horses are mixed at racing, training, or breeding facilities, their origin of travel can impact the potential for an outbreak. During an outbreak at the Hong Kong Jockey Club in 1992, individual or groups of animals were identified that had recently arrived from the UK and Ireland, countries where horses encounter EIV and, accordingly, are routinely vaccinated. Horses from Australia and New Zealand, where EIV is not prevalent, were stabled at the same facility. These horses had not been vaccinated against EIV until their arrival at the race-track facility. The incidence of disease was much higher (52%) among the horses from Australia and New Zealand as compared to those from the UK and Ireland at (20%). This outbreak represents an example of the conditions where equine influenza can spread among a susceptible population of horses. Thus, it appears that the origin and destination of horses were key to understanding the risk factors associated with the 1994 epidemic at the Honk Kong Jockey Club.

Impact to the Industry

Horses infected with the EIV typically are symptomatic but recover with good management. Why then is there such concern over this disease in the equine community? If the horse population is categorized into those that are "recreational animals" and those that are "performance animals", one can better appreciate the impact of influenza. It is the economic loss

associated with influenza outbreaks in performance animals that makes the disease important. With the expansion of international trade in horses and equine products (semen), comes the associated economic significance, mirrored by the potential spread of infectious agents. With the exception of humans, horses travel internationally (usually by air) more than any other animal species (Timoney, 2000). Because of the potential risk of reduced or lost performance of racing, jumping, eventing, show, or breeding equids, much emphasis has been placed on monitoring and controlling infectious upper respiratory disease (IURD) in horses. Thus, to appreciate the impact of influenza on the equine community, both performance and economics can be considered.

Horses that are sub-clinically infected with EIV not only cause endemic disease, but also are at risk of training or performance failure. Movement of sub-clinically infected horses at the international level provides the potential for interaction with susceptible populations (Timoney, 2000). As with other infectious diseases, vaccination strategies are always of primary importance. Current methods for vaccination, while moderately effective at controlling clinical illness and the spread of infection, do not provide a tool for complete eradication (Mumford, 1999a). This is due to not only the type of vaccines routinely used, but also related to the continuous antigenic changes that occur with influenza virus. Because of this, individual events of URD associated with EIV are frequent and result in the loss of performance. The second most common cause of loss of training days for racehorses is a result of URD from various etiologies (Wood, Newton et al., 1999). Outbreaks of IURD resulting from infection with equine herpes virus-1 and 4 (EHV) and EIV can occur when horses are congregated at places such as a "pinhooking" facility. The spread of IURD among yearlings can cause a significant decrease in performance associated with training failure. In a recent survey, three out of 76 thoroughbreds (4%) followed at a pinhooking facility in Ocala Florida were diagnosed with clinical signs of IURD (Hernandez, J. personal communication).

A second impact of EIV is the loss of revenue associated with an outbreak. In 1975, the cost resulting from infection with EIV was estimated to be $668 per horse (Bryans, 1975). Watkins reported the loss of revenue in a more recent outbreak during the 1992 racing season at the Hong Kong Jockey Club. The cancellation of seven race meetings resulted in the estimated loss of nearly $ 1billion US dollars, of which, 15% would be collected in the form of tax revenue to the local community (Watkins, 1997). To further appreciate the economics associated with horse racing, Table 1-2. illustrates an example of the 1995/96 racing season in Hong Kong.

TABLE 1-2. Economic losses associated with an outbreak of EIV at the Hong Kong Jockey Club during the 1995/1996 racing season

- 69 race-meetings
- 517 races
- 6,090 starters from an average population of 1,000 horses
- US $ 92 billion turnover for the season
- US $ 130 million average turnover every race meeting
- US $ 17.5 million average turnover every race
- US $ 1.5 million bet on average on every horse that raced
- US $ 1.3 billion returned to the community in tax, duty and donations
- Total attendance of 3,270,100 with an average of 47,400 every racemeeting
- Total employees of 16,178

Taken from Watkins, 1997

Vaccination Against Equine Influenza Infection

Vaccine Theory

Vaccines have been developed in an attempt to eliminate or control the spread of infectious agents as well as to moderate clinical disease. Vaccination not only eliminates some discomfort to the animal, but has been shown to be a cost-effective way to minimize economic

losses as well. Vaccination, at the most basic description, is the delivery of an immunogenic antigen (live, killed, or parts of a pathogenic organism) to the host resulting in a protective immune response. The ideal vaccine should be efficacious, economic to manufacture and administer, and have minimal adverse side effects. A great deal of progress in the fields of genetics, virus structure, and the immune system has allowed the development of effective vaccines. New approaches to vaccines used in veterinary medicine must result in those that minimize the undesirable side-effects, are efficacious, and safe to the vaccinate well as non-vaccinated animals and humans.

There has been a great deal of progress in the standardization of vaccine testing relative to EIV. Vaccines against EIV have a direct correlate between HA content and the level of protection. Because of this, the hemagglutination inhibition assay has been a gold standard to determine the efficacy of vaccines (Hoskins, 1967). Another reliable in-vitro measure of serum antibody is the single radial hemolysis (SRH) assay (Wood, Schild et al., 1983). Both have been used extensively during the development of vaccines against EIV.

Early versions of vaccines against EIV included inactivated virus vaccines with various adjuvants. Inactivated vaccines are still in wide use today. However, inactivated-virus vaccines provided incomplete and short-lived protection against severe clinical disease. Furthermore, they are unable to prevent infection. While improvements have been made with adjuvants that result in a prolonged antibody response, alternative methods to present antigen in a manner that more closely resembles natural infection are needed. In an effort to mimic complete protection provided by natural infection, novel approaches in vaccine development have included modified-live virus (MLV) and recombinant DNA vaccines. Presently, vaccine development, along with international efforts to improve surveillance are identified as priority issues for the control of EIV (Mumford, 1999a).

Killed Vaccines

Currently, inactivated-virus influenza vaccines are licensed for parenteral administration in humans (Fields, 1996) and animals (Horzineck, Schijns.V.E. et al., 1997). A killed or inactivated vaccine constitutes an infectious agent which has been altered by chemical or other methods, such that replication is not possible, yet antigenically important proteins are conserved. Whole virus and subvirion vaccines contain intact, inactivated virus, or purified virus disrupted with detergents that solublize the lipid-containing envelope. Unfortunately, some inactivating compounds (formalin or beta-propriolactone) can alter the immunogenicity of the antigenic proteins (Duque, Marshall et al., 1989).

One of the greatest shortcomings of inactivated vaccines is an incomplete immune response. While inactivated-virus vaccines can elicit a good humoral response, they are not effective at inducing cell-mediated or mucosal immunity. However, the use of various lipid containing structures or immune stimulating complexes (ISCOMs) may produce both a local and humoral response when used with inactivated vaccines (Rimmelzwaan & Osterhaus, 1995).

Immune-mediated resistance induced by experimental or natural infection with influenza virus is based on the level of protective HA antibodies. The same level of protection is not achieved with the use of inactivated-virus vaccines. This is of particular importance in humans and horses where HA surface antigens contained in a vaccine may not be effective against the strain circulating in an epidemic (Mostow, Schoenbaum et al., 1970).

Live Vaccines

Live vaccines have several distinct advantages over those that are inactivated. They possess properties that allow various routes of inoculation, they present a complete suite of viral antigens, they can induce cell mediated and humoral responses, and they are economical to produce relative to the cost of inactivated-virus vaccines. Live vaccine are not without drawbacks, however. The associated disadvantages include immunosuppression and the potential

to cause and spread disease among immunocompromised individuals. Modified live virus vaccines have been the subject of experimentation in humans for use as an immunoprophylaxis against influenza for several years (Advisory Committee on Immunization Practices (ACIP), 1999).

The exact mechanism by which a virus becomes attenuated during serial passage is not known. However, the genes responsible for the production of toxins, structural proteins, and nucleic acid metabolism can be altered, and result in attenuation (Muster, Subbarao et al., 1991). One of the potential problems with attenuated viruses is their potential to revert back to a virulent form. In some vaccines, attenuation is the result of point mutations that can revert back upon a single passage in vivo (Minor, John et al., 1986). An example of this occurred during a study that used the H3N8, A/equine/Miami/63 virus at the 6^{th} passage as a challenge-strain in horses. The virus was passaged five times and inoculated into two sero-negative ponies. Although virus replication occurred, neither pony showed clinical signs. The virus from one of the ponies was inoculated into the nasopharynx of a third seronegative pony and clinical disease ensued accompanied by fever and coughing. The same virus strain was again isolated from the horse and passaged once in eggs. A subsequent challenge using the egg isolate at 10^8 EID_{50}/mL was capable of inducing clinical signs (Mumford, Wood et al., 1988).

New approaches in the development of live vaccines for influenza must take antigenic drift into account. For this reason, it is not practical to produce an attenuated form of each subtype that emerges during an outbreak. To address this problem, site-directed mutagenesis is used to produce an attenuated virus with changes in genes such as NA (Muster, Subbarao, Enami, Murphy, & Palese, 1991). In one study, a wild-type virus was passaged using a method that resulted in a mutation. The attenuated virus was mixed with a wild-type virus and the reassortant progeny proved to be effective and safe (Edwards, Dupont et al., 1994).

Recombinant DNA Vectors

A recombinant DNA vaccine will typically consist of an avirulent organism that is constructed by site-directed mutagenesis to carry a gene insertion encoding for an immunogenic antigen. This approach also assumes the role of the host to replicate the vector organism (Perkus, Piccini et al., 1985). A safe and effective virus vector will possess a large genome consisting of non-essential genes that can be deleted. Furthermore, the gene-deletions should result in a non-virulent virus that retains the ability to replicate. As knowledge about gene insertions and deletions grows, so will the number of vaccines that carry this technology. Large DNA viruses such as vaccinia virus are suited for this construction (Cooney, Collier et al., 1991). One study included a vaccinia virus and a plasmid vector used to express surface glycoproteins encoded by two strains of EIV types 1 and 2 (Beverly, Brown et al., 1988). In this study, a recombinant vaccinia EIV vaccine was used that expressed HA and NA. They found that an initial serum antibody response rapidly declined in manner similar to that seen in naturally infected horses. They also concluded that, in addition to immunoglobulin production, cytotoxic T lymphocytes (CTL) are partially responsible for the protection provided by a recombinant vaccinia virus vaccine. In similar studies, hamsters and mice were reported to have an increased CTL response following inoculation with a recombinant vaccine expressing A/Japan/305/57 antigen (Smith, Mackett et al., 1983).

Immune Response

The impetus for generating an antigen-specific response that provides protection against the harmful effects of a pathogen (vaccination) assumes the basic requirement of a functioning immune system. The induction of a protective response is referred to as acquired immunity. A second form of protection comes from innate or natural immunity which describes non-immune barriers such as respiratory mucous, mucociliary transport and the cough reflex (Dixon & McGorum, 1997). Vaccines are effective at generating acquired immunity against a pathogen.

The type of immune response is dependent upon the preparation of the antigen and can be either cell-mediated or systemic or a combination of both.

Antigen Presentation

More specifically, the nature of the immune response is dependent upon the way antigen is processed and presented by antigen presenting cells (APC) for recognition by specialized effectors cells. This is the basis for the increased efficacy of whole-virus preparations over that of inactivated or subunit vaccines. Studies have shown that vaccines containing MLV induce several appropriate effector mechanisms that lead to a comprehensive response (Lunn, 1997). Antigen in its native form is not recognized and acted upon by all immune cells. B cells recognize native antigen through a receptor associated with immunoglobulins on their surface. In contrast, T cells responsible for functions related to cytotoxicity and antibody production, require antigen to be presented by specialized APC which process antigen into recognizable peptide segments. Examples of APC are monocytes, macrophages, B cells, and dendritic cells (Dixon & McGorum, 1997). Before T-cell recognition can occur, antigen peptides must be associated with major histocompatibility complex (MHC) molecules through either an endogenous (MHC I) or exogenous (MHC II) pathway. The subtypes of T cells that respond to antigen include $CD4^+$ T helper (Th) via the MHC class II exogenous pathway or $CD8^+$ CTL through the MHC class I endogenous pathway (Sprent & Tough, 1994;Accolla, Auffray et al., 1991). Virus-infected cells will process viral-peptide fragments that are expressed on the surface in association with MHC class I class molecules resulting in a Th1-like response (Monaco, 1992). This results in the differentiation and activation of CTL effector cells and T helper lymphocytes that, in turn, stimulate antibody-producing cells (Dixon & McGorum, 1997). Typically, influenza antigen that is presented in an inactivated-virus vaccine does not enter the endogenous pathway and is unable to activate $CD8^+$ T cells. Instead, viral peptides are presented on the cell surface in association

with MHC class II molecules resulting in, primarily, a Th2 response and antibody production (Rimmelzwaan & Osterhaus, 1997).

Duration of Antibody Post-infection or Vaccination

Vaccines are based on the presentation of parts or whole pathogenic organisms to induce an immune response. Protection against severe clinical disease during infection with influenza is based on the formation of antibodies to HA and NA surface glycoproteins. Cell-mediated immunity is typically directed toward the recognition of the NP of the influenza virus and is also believed to play an important role in recovery after infection. Despite the importance of both humoral and cell-mediated immunity, the primary surrogate of protection is the measure of short and long-term circulating immunoglobulin proteins. IgG and IgM can provide protection by neutralization of the virus through antigen-specific binding, opsinization to enhance the removal of antigen by phagocytes, and the activation of complement (Dixon & McGorum, 1997).

Historically, the standardization of protocols to determine the efficacy of influenza vaccines has been limited by the variability in assay methods. One study compared the ability of the HI and SRH assay to detect a dose-dependant response of a vaccine against EIV (Wood, Schild, Folkers, Mumford, & Newman, 1983). This study confirmed the greater specificity of SRH over that of the HI test. Antibody titers were low and undetected by HI after a primary inoculation with a bivalent vaccine containing inactivated A/equine/Prague/56 and A/equine/Miami/63. However, low levels of SRH antibody were detected after the first vaccination and a significant rise was detected after a booster inoculation. Another study correlated HA units/dose and antibody titers associated with protection against severe disease (Mumford, Wood, Folkers, & Schild, 1988). The results were confirmed with a SRH test which showed a correlation between protection against clinical disease and a pre-challenge minimum zone of hemolysis equaling 74 mm^2.

In a more recent study, ISCOMS were found to be effective as an adjuvant during vaccination against EIV challenge (Mumford, Jessett, Dunleavy, Wood, Hannant, Sundquist, & Cook, 1994a). Two vaccines, consisting of HA antigen from A/equine/2/Newmarket/77 or A/equine/2/Brentwood/79, were complexed with an ISCOM adjuvant (tetanus toxoid adsorbed on aluminium phosphate). A dose-related response established that a minimum of 15 micrograms of HA/dose would provide a protective and slower declining serum antibody titer. Antibody titers associated with protection were not achieved until after the third inoculation of vaccine, but were maintained for up to 15 months. The use of the ISCOMs increased antibody-mediated protection against clinical disease. However, while local immunity and cell-mediated immunity were not investigated in this study, they may have contributed to the resulting increased protection. The use of ISCOM adjuvants has been shown to facilitate the presentation of antigen in association with MHC class I molecules to T-cell receptor (TCR) molecules on CTL. The use of ISCOMs will result in local antibody production which is most likely the most important mechanism of prevention (Horzineck, Schijns.V.E., & Denis, 1997). As stated previously, even though antibody production is considered key to protection , several reviews highlight the importance of the local and cell-mediated response during EIV infection (Lunn, 1997; Hannant and Mumford, 1997; Plateau et al., 1997; Dale et al., 1997; Mumford et al., 1983; Mumford et al., 1994).

Mucosal Immunity

The mucosa of the respiratory tract is lined with tissue responsible for protection against foreign antigens as the first line of defense (Small, 1990). Mucosal immunity is important in preventing infection through local antibody and cell-mediated responses (Bender & Small, Jr., 1992). In the mucosal lining, antigen is taken up by specialized epithelial (M) cells which mediate its transport to mucosal-surface-associated lymphoid tissues (MALT) (Horzineck, Schijns.V.E., & Denis, 1997;McGhee, Mestecky et al., 1992). T-lymphocyte helper cells are thought to be the source of localized cytokines IL-5 and IL-6 involved in class switching

(Horzineck, Schijns.V.E., & Denis, 1997;McGhee, Mestecky, Dertzbaugh, Eldridge, Hirasawa, & Kiyono, 1992). Within the MALT, antigen presentation will result in class switching of immunoglobulins and the activation of IgA-specific memory B cells. IgA secreted at the mucosal surface will effectively bind foreign antigen to prevent attachment to the epithelial cell surface (Holmgren, Czerkinsky et al., 1994). In addition to the neutralization of virus in the lumen by IgA, the transcytosis system has two additional effector mechanisms. IgA can effectively neutralize viruses within the epithelial cell as well as the lumenal surface (Mazanec, Coudret et al., 1995a). Intracellular neutralization of virus has been demonstrated experimentally by infection of Madin Darby canine kidney cells (MDCK) with influenza or Sendai virus. There is evidence that IgA can inhibit viral assembly (including influenza) within the epithelial cell by binding viral proteins released from the Golgi (Mazanec, Nedrud et al., 1993). Newly synthesized viral glycoproteins HA and NA move from the trans-golgi network to the lumenal surface via endosomal compartments containing polymeric IgA (pIgA). Yet another mechanism of protection mediated by IgA molecules is the inter-cellular transport of antigen across the luminal surface of epithelial cells coupled by a secretory component (Mestecky & McGhee, 1987).

Again, an important component of vaccine-strategy is to induce a comprehensive immune response, including the production of local IgA. In mice (Tamura, Funato et al., 1990;Tamura, Funato et al., 1991) and humans (Boyce, Gruber et al., 2000;Tomoda, Morita et al., 1995), the production of nasal IgA antibody has been associated with the use of MLV vaccines and infection. In contrast, intramuscular administration of inactivated virus preparations do not result in nasal IgA antibody production (Clements, Betts et al., 1986). In the horse, local IgA antibody production was not detected after immunization with an MLV vaccine (Lunn, Hussey, Sebing, Rushlow, Radecki, Whitaker-Dowling, Youngner, Chambers, Holland, Jr., & Horohov, 2001). Furthermore, similar to humans and mice, the use of an inactivated-virus vaccine in horses does not induce nasal IgA formation (Nelson, Schram, McGregor, Sheoran, Olsen, & Lunn, 1998).

Mucosal inoculation with a DNA vaccine, containing A/equine/2 proteins, resulted in the production of nasal IgA antibody in ponies (Lunn, Soboll, Schram, Quass, McGregor, Drape, Macklin, McCabe, Swain, & Olsen, 1999).

Hypothesis and Objectives

Hypothesis

The intranasal or intramuscular inoculation of a novel recombinant DNA vaccine constructed of an EHV-4 vector encoding HA and NA genes from A/equine/2/Kentucky/94 for antigen presentation will induce systemic and local antibody production as well as the cell-mediated arm of the immune response. The recombinant vaccine will provide protection against infection in the form of reduced virus shedding and clinical disease.

The goal of this study is to show that the use of a novel recombinant DNA vaccine will provide a similar immune response compared to that seen after natural infection or immunization with a cold-adapted modified-live virus vaccine. Furthermore, immunization with the recombinant vaccine will result in a greater decrease in viral shedding and clinical signs over that seen after immunization with an inactivated-virus vaccine upon challenge-infection.

Objectives

The objectives of this study are: 1) to further develop existing techniques to sample and assay horses for the presence of serum and nasal antibodies and cell-mediated immunity to EIV, 2) conduct animal studies to determine the immune response of horses after immunization with either a recombinant DNA, inactivated-virus, or modified-live virus (MLV) vaccine, 3) determine the ability of each vaccine to eliminate or reduce virus shedding and clinical disease, and 4) compare the vaccine-induced response to that seen in horses that are experimentally infected with EIV.

CHAPTER 2

STANDARDIZATION OF IN-VITRO TECHNIQUES TO STUDY THE IMMUNE RESPONSE IN HORSES

Introduction

Studies related the development and testing of vaccines against influenza have incorporated a number of methods to sample and assay for surrogates of protection. These have included serology, nasal antibody, cell-mediated immunity, virus shedding, and clinical diagnosis (Asanuma, Aizawa et al., 1998;Bender, Johnson et al., 1991;Hannant, Easeman, & Mumford, 1999;Hannant & Mumford, 1989;Lunn, Soboll, Schram, Quass, McGregor, Drape, Macklin, McCabe, Swain, & Olsen, 1999;Mumford, Wood, Folkers, & Schild, 1988). Previous studies have shown a correlation between the formation of antibodies and protection against severe clinical disease in horses. Serum antibodies have been recognized as a surrogate marker of protection and are the basis for measuring vaccine efficacy against infection with EIV. Therefore, various assays were used to confirm the response of horses to vaccination and infection. Hemagglutination inhibition, SRH, and neutralization assays are regarded as the "gold-standards" for measuring serum antibody in response to influenza infection. These assays were used as reference assays during the standardization of an ELISA. Optimization of the ELISA will include virus growth and purification, determination of sensitivity and specificity, and further development of existing techniques to sample and assay for nasal antibodies. Sensitivity and specificity of an assay relates to the probability of testing positive or negative while that is, in-deed, the true condition. In the present study, sensitivity and specificity of the ELISA were used to determine a "critical" or cutoff value associated with seroconversion. Here, a "Critical-Value"

plot, similar to those used in Receiver-Operator Characteristics (ROC) plot analysis was used to graphically display the sensitivity and specificity of the ELISA at various critical or cutoff values.

Several reports have included CMI analysis to characterize its importance in mice (Bender, Johnson, & Small, 1991;Bennink, Yewdell et al., 1984;Epstein, Lo et al., 1998), humans (Bernstein, Gardner et al., 1998;Kruse, Moriabadi et al., 2001), cats (Song, Collisson et al., 1992), cattle (Abdy, Howerth et al., 1999), and horses (Ellis, Bogdan et al., 1995) (Ellis, Steeves et al., 1997;Hammond, Cook et al., 1997)during their recovery from viral infections. Cell-mediated immune function tests include lymphocyte proliferation, cytotoxic T-lymphocyte assays, and cytokine determination. The lymphocyte proliferation assay has been routinely used in human immunology as an indicator of basic immunocompetence (Fletcher, Klimas et al., 1992). The lymphocyte proliferation assay is a good indicator of immune function in general, or to a specific pathogen, but often interpretation of results is difficult due to variability in the data. As with humans, this is the case with horses (Ellis, Bogdan, & Kanara, 1995). The methods used to collect, cryopreserve, culture, and stimulate lymphocytes can vary. Recent studies characterizing the blastogenesis of cells after stimulation have included the use of a tetrazolium dye (Owen's reagent) (Behl, Davis et al., 1994;Lappalainen, Jaaskelainen et al., 1994). This method has been compared to and used in place of a [^{3}H] thymidine uptake assay (Wong & Goeddel, 1994). In that study, the tetrazolium dye method demonstrated comparable sensitivity to the [^{3}H] thymidine uptake assay. Due to the advantages of using a non-radioactive reagent, the ability of the tetrazolium dye to detect increases in equine PBMC numbers during a proliferation assay was investigated.

The role of cytokines in the regulation of immune function during viral infection has been described elsewhere (see review) (Guidotti & Chisari, 2000). Limited information is available on the characterization and generation of monoclonal antibodies to equine cytokines (Horohov, 1999;Lunn, Sobol et al., 1999) and ELISA techniques to measure cytokine production in horses

have not been described. However, studies are ongoing that describe the development and future application of these reagents (Lunn, Sobol, Swiderski, Horohov, & Olsen, 1999). Generation of primers to detect equine cytokine mRNA expression in a PCR assay has been described (Giguere & Prescott, 1998). Furthermore, primers for equine cytokine mRNA have been constructed for use in "Realtime" PCR assays (Giguere & Prescott, 1999). In the same study, Giguere reports on the increased sensitivity of Realtime methods over that of conventional PCR.

In the current study, HI and SRH assays are used as reference assays during the standardization of ELISA techniques. To evaluate the diagnostic performance of the ELISA to detect serum and nasal antibodies, methods were standardized to grow and purify EIV, determine the sensitivity and specificity, and develop sampling techniques. Due to various protocols reported elsewhere, methods to store, culture, and stimulate equine peripheral blood mononuclear cells (PBMC) were optimized. In addition, Realtime PCR methods were developed to investigate the expression of equine cytokine mRNA resulting from in-vitro stimulation of PBMC with EIV antigen.

Materials and Methods

HI Assay

Hemagglutination inhibition (HI) assays were completed as previously described (Hsiung, 1994). The virus stock used for hemadsorption of test sera was Kentucky-95. Positive and negative control reference sera were obtained from the National Veterinary Services Laboratory (NVSL) (Ft. Collins, CO). Allantioc fluid containing live virus was treated with diethyl-ether at a 1:1 ratio for 15 min. The aqueous portion of the extract was removed and used in test-wells of a v-bottom, 96-well plate. At the end of the last incubation period, plates were tilted at a 70° angle and positive inhibition was determined by the absence of "streaming" of chick RBC in the test wells. Hemagglutination inhibition titers were reported as the number of

hemagglutinating units (HAU) per 25 µl of serum. A 1:128 HI titer indicates a dilution factor of 128 to obtain 1 HAU.

SRH Assay

The measurement of serum antibody levels to EIV was conducted using single radial hemolysis techniques as adapted from the European Pharmacopoeia Commission. Sheep red blood cells (SRBC) were sensitized with EIV Kentucky-95 at an HA titer of not less than 1:64 per 25 µl. Guinea pig complement was added to sensitized SRBC which were then added to 1% agarose at 42° C. The mixture was poured onto plastic plates (ICN Biomedical Inc., Costa Mesa, CA) marked with mm increments for measuring the zone of hemolysis. Once plates had cooled to room temperature, 3-mm-diameter holes were punched into the gels and the plates were stored at 4° C until used. Plates were used not more than three dy after they were made. A negative control plate was made as described without the addition of virus.

Sera were heat-inactivated at 56° C for 30 min. Ten µl of heat-inactivated test serum was pipetted into the 3-mm wells and incubated for 20 hr at 34° C. After the incubation period, the diameter of the clear circular area indicating hemolysis was measured. The zone of hemolysis was calculated and expressed as mm^2 using the following formula:

Zone of hemolysis = $\pi r^2 - 7mm^2$

where r = radius of the hemolyzed circle.

ELISA Standardization Techniques

Virus purification for use in ELISA

Virus was purified by a procedure adapted from a previously published technique (Laver, 1969). Kentucky-95 EIV was grown in ten-dy-old embryonated chick eggs for 72 hr at 34° C. Allantoic fluid containing EIV at an HA titer of 1:256 was harvested from eggs and clarified at

4,500 rpm for 30 min at 4° C. EIV was loaded into Beckman ultra-clear centrifuge tubes and pelleted by centrifugation in a fixed angle (70.1 Ti, Beckman) rotor at 50,000 x g (27,000 rpm) for 90 min at 4° C. The "slurry" pellet at the bottom of each tube was re-suspended in 50 µl of cold TSE buffer and held on ice until pooled. Virus was pooled in a total of 3 mL of cold TSE buffer. 3 mL of the concentrated EIV (1:1024 HA) was layered onto a 30%-60% discontinuous sucrose gradient in Beckman ultra-clear centrifuge tubes, placed in a pre-chilled swing-bucket (41-Ti, Beckman) rotor, and centrifuged at 95,000 x g (30,000 rpm) for 120 min at 4° C. After centrifugation, a opaque cloudy band of virus was visualized by holding the tube against a black background. A 22-ga needle on a 3-mL sterile syringe was used to puncture the side of the tube and aspirate the cloudy material. To inactivate the virus prior to use on ELISA plates, virus was adding to Sarcosyl at a final dilution of 0.5% in TSE buffer. The virus and sarcosyl was incubated for 15 min on ice. The virus was then washed by further dilution in 9 mL of TSE and centrifuge as previously described at 35,000 rpm for 90 min at 4° C. The purified, inactivated virus was re-suspended in cold coating buffer containing 0.1% final concentration of sodium azide (NaN_3).

ELISA development

Immunlon-2 HB, 96-well microtiter plates (Dynex Technologies, Chantilly, VA) were coated with gradient-purified EIV Kentucky-95 diluted in a coating buffer (see appendix B) to 10 HAU per well and incubated at 4° C overnight. The plates were washed 3 times with a wash-buffer (see Appendix B). The plates were blocked with a 1% fish-gelatin blocking buffer (see Appendix B) for 2 hr at 37° C, washed 3 times in wash buffer, and stored at 4° C until used. All test sera, nasal secretions, and reagents (anti-immunoglobulins) were diluted in a 1% fish-gelatin buffer. Test and control sera were diluted at 1:1000 in fish-buffer. Prior to testing, nasal secretions were treated with a 1:1 mixture of 10 mM 1-4 dithiotreatole (DTT) (Sigma Chemical Co., St. Louis, MO) for one hr at 37° C to eliminate non-specific binding of mucous proteins to

the test wells. The nasal secretions were then diluted to a final concentration of 1:300 in 1% fish buffer. Positive and negative controls were diluted at the same ratio for each test sample. Positive-reference-serum was obtained from NVSL in Ames, IA. Pathogen-free herd serum was used as negative-control sera (Sigma Chemical Co., St. Louis, MO). A positive control for IgA was obtained by collecting nasal secretions from a horse that had been experimentally infected with live EIV Kentucky-95. This horse was determined to be serum-negative to EIV Kentucky-95 and 94, Newmarket-96, and Prague-56 prior to infection and serum-positive to Kentucky-95 afterwards as determined by HI assay. Nasal secretions from horses that were serum-negative by HI assay for EIV Kentucky-95 and 94, Newmarket-96, and Prague-56 were used as a negative control sample source.

Fifty µl of test samples and control were added to triplicate wells of a 96-well plate. Plates were sealed with an adhesive plate cover and incubated overnight at 4° C. For serum samples, plates were washed three times and 50 µl of a mouse, anti-horse IgGa (CVM-45) monoclonal antibody diluted to 1:40 in fish buffer was added to each well. Plates were incubated for two hr at 37° C. Plates were washed 3 times and 50 µl of secondary goat anti-mouse IgG + IgM H+L chains (minimal cross-reactive to human, bovine and horse serum proteins) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted in fish buffer to 1:5000 was added to each well. After a two-hr incubation at 37° C, the plates were washed prior to the addition of 100 µl O-phenylenediamine dihydrochloride (OPD) (Sigma Chemical Co., St. Louis, MO) to each well followed by 50 µl of a 3M HCl stop solution. Color development took approximately ten min. The plates were read immediately at 490 nm using a Revolution, ELISA plate reader (Dynex Technologies Inc., Chantilly VA). Nasal secretion samples were handled in the same manner as the serum samples. However, the primary antibody anti-horse IgA (BVS-1) was diluted at 1:40 and the secondary antibody was diluted at 1:2000 in fish buffer. Results were reported as an ELISA index.

IgGa (CVM-45) and IgA (BVS-1) were extensively characterized during the Second International Equine Leukocyte Antigen Workshop, Squaw Valley, California, July 1995. The specific workshop antibody number is indicated in parentheses (Lunn, Holmes et al., 1998).

To standardize the test results between plates, the ELISA data were reported as an "ELISA Index" calculated by using the following formula:

ELISA Index = OD reading of test serum/OD reading of positive control serum

Sensitivity and Specificity of the ELISA

The "critical value" or cut off point, where an animal is considered sero-negative to EIV, is based on criteria that are related to the sensitivity and specificity of the ELISA under standardized conditions established in our laboratory.

The cutoff value was determined for the ELISA using methods previously described. Briefly, Immulon II HB plates (Dynex Technologies Inc.) were coated with 10 HAU per well of gradient purified Eq/2/Ky/95 EIV. Standardization and optimization of reagents were completed by adjusting dilutions of control and test sera (1:1000), primary antibody against equine IgGa (1:40), and peroxidase-conjugated secondary antibody (1:5000). Serum samples from 30 horses that were either seronegative or those that had been experimentally infected and had antibodies to EIV were used. Serological evidence of exposure to EIV, as determined by HI and SRH assays and a known history of experimental infection, were used as inclusion criteria for samples. Sera from horses that had been infected by aerosolized chick allantoic fluid containing approximately 10^{6-8} EID_{50} and had HI titers higher than a 1:64 dilution and SRH titers higher than 75 mm^2 were used as positive controls. Sera with no known previous exposure to EIV or vaccines to EIV and had HI titers of less than 1:8 and no detectable SRH titers were used as negative controls.

Samples were randomly ordered and labeled using coded numbers generated from an Excel software program. Triplicate wells containing positive and negative samples were run on

two, 96-well micro-titer plates on the same day. Plates were treated as described above and OD readings were collected on test samples using an ELISA reader at 490 nm.

Data was used in 2 x 2 tables to determine the sensitivity and specificity of the ELISA at various "critical or cut-off values". The critical value represents the OD reading generated by the ELISA that correlates with seroconversion. Sensitivity and specificity were confirmed and represented constructing a "Critical-Value" plot similar to that used for Receiver-Operating Characteristic (ROC) plot analysis (Zweig & Campbell, 1993). In the present study, a decision threshold represented the point at which the ELISA was able to detect antibody in serum samples. This is referred to as the "critical-value" and was used to establish a reference value at which horses were considered to seroconvert after vaccination or infection.

Lymphocyte Proliferation Assay

Proliferation assays were adapted from methods described elsewhere (Ellis, Bogdan, & Kanara, 1995;Hannant & Mumford, 1989;Hannant, 1994). PBMC were collected and isolated from whole blood using methods described elsewhere (see sample collection, chapter 4). Frozen PBMC were thawed rapidly in a 37° C water bath and washed twice by centrifugation at 250 x g for ten min at 4° C in PBS. The cells were re-suspended in warm RPMI-1640 with 10-mM Hepes buffer, 2-mM L-glutamine, 0.075% w/v sodium bicarbonate, 1 mM sodium pyruvate, 100 U/mL penicillin-G sodium, 100 μg/mL streptomycin, 10% BFS (Gibco BRL, Grand Island, NY), and recombinant human Interleukin-2 (rh IL-2) at varying concentrations Units/mL (Sigma Chemical Co., St. Louis, MO), and then cultured overnight before antigen and mitogen stimulation. Cells were adjusted to 1.5 x 10^6 cells/mL and added to a 96-well, U-bottom plate at 100 μl per well. Proliferation assays were conducted to determine the effects of varying the concentration of rh IL-2, and the infectious-dose of EIV. Blastogenesis was measured in response to heat-inactivated (57° C for 30 min), UV-inactivated (Newton, Wood et al., 1999), and live

A/equine/2/Kentucky/95 and 91 EIV. The A/2/Kentucky-91 strain of EIV (contained in the MLV vaccine) was cultured in ten-dy-old embryonated chick eggs (HA titer of 1:64) and frozen at –80° C, prior to its use in the proliferation assays. To compare the response of PBMC stimulated with live EIV to those stimulated with inactivated antigen preparations, cells were cultured with live virus (EID_{50} $10^{8.3}$) for 45-min, washed two times, and resuspended in CM. The cells were cultured for four dy at 37° C and 5% CO_2. Positive controls included stimulation with Concanavalin A (Con A) (Sigma Chemical Co., St. Louis, MO) at 5 μg/mL and pokeweed mitogen (Gibco BRL. Grand Island, NY) at 4 μg/mL. Negative controls included media alone and allantoic fluid without virus. Cells were incubated at 37° C at 5% CO_2 for 96 hr. The antigen specific and mitogen induced proliferation response was measured by using the Cell Titer 96™ Non-Radioactive Proliferation Kit (Promega, Madison, WI). This method is based on the addition of a tetrazolium salt dye ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfolphenyl)-2H-tetrazolium (Owen's reagent) that is subsequently converted into a colored formazan product detectable by an ELISA reader. At the end of a four-hr incubation at 37° C and 5% CO_2. Color development resulting from the formazan product was measured at 570 nm using a Revolution ELISA plate reader (Dynex Technologies, Chantilly, VA). A stimulation index was calculated as follows where the un-stimulated cell control represents cultures incubated in media alone:

$$\text{Stimulation Index (SI)} = \frac{\text{Absorbance of stimulated cells - Absorbance of medium}}{\text{Absorbance of un-stimulated cells - Absorbance of medium}}$$

To confirm the sensitivity of the ProMega Cell Titer 96™ Non-Radioactive Proliferation Kit, duplicate cultures of PBMC stimulated with Con A and EIV antigen were assayed to determine the proliferation response using radioactive [^{3}H] thymidine uptake as well as the tetrazolium dye method. To determine potential changes in the viability of equine PBMC after freezing, cells were assayed as described above prior to and after cryopreservation in liquid nitrogen. Prior to viability staining and the addition of mitogen or EIV, frozen cells were thawed

and allowed to incubate overnight in three-mL, Falcon polyethylene tubes (Becton Dickinson, Franklin Lakes, NJ) at 37° C and 5% CO_2. This allowed the leaching of DMSO used during cryopreservation.

Cytokine mRNA Determination in Equine Peripheral Blood Mononuclear Cells
RNA isolation and reverse transcription

Prior to viability staining and the addition of EIV antigen, frozen cells were thawed and allowed to incubate in three-mL, Falcon polyethylene tubes (Becton Dickinson, Franklin Lakes, NJ) overnight at 37° C and 5% CO_2. Lymphocytes were cultured in 24-well plates at 5 x 10^6 to 1 x 10^7 cells/well. The cultures were stimulated with 128 HAU of heat-inactivated EIV Kentucky-95 and incubated for 72 hr at 37° C and 5 % CO_2. Afterwards, cells were washed twice in PBS and total RNA was isolated by the use of a Rneasy® Mini Kit (Qiagen, Valencia, CA). All RNA samples were treated with amplification grade DNAse I (Gibco BRL, Rockville, MD) to remove any traces of genomic DNA contamination. Briefly, 1 U of DNAse I and 1 µl of 10 X DNAse I reaction buffer were mixed with 1.5 µg of total RNA in a ten µl reaction. The mixture was incubated for eight min at room temperature and then inactivated by adding one µl of 25 mM EDTA and heating at 65 °C for ten min.

cDNA was synthesized with a Clontech 1st strand cDNA synthesis kit (Clontech, Palo Alto, CA) by the method recommended by the manufacturer. Briefly, one µg of total RNA was mixed with one µl of oligo $(dT)_{18}$ primer (20 µM) and heated at 70° C for two min. After the mixture was cooled to room temperature, the following reagents were added in the order listed: four mL of 5X reaction buffer (250mM Tris-HCL [pH 8.3], 375 mM KCL, and 15 mM $MgCl_2$), one µl of deoxynucleoside triphosphates (10 mM each), 0.5 µl of Rnase inhibitor (40 U/µl), and one µl of Moloney murine leukemia virus reverse (MMLV) (200 U/µl). The mixture was incubated at 42° C for one hr, heated at 94°C for 5 min, diluted to final volume of 100 µl, and stored at –70° C until used for PCR analysis.

cDNA in reverse transcription products was confirmed as previously described (Giguere & Prescott, 1998). Briefly, two μl of cDNA was amplified using primer pairs for equine β actin (Clontech, Palo Alto, CA) in a 25-μl PCR assay in the presence of 0.4 μM of each primer, 0.2 μM (each) of deoxynucleoside triphosphates, five μl of 10X reaction buffer (containing 10 mM TrisHCl [pH 8.3] and 50 mM KCl), 1.5 mM $MgCl_2$, and two U of *Taq* DNA polymerase (AmpliTaq; Perkin-Elmer, Branchburg, N. J.). Polymerase chain reactions were performed with an initial denaturation step at 94° C for 2 min and 35 cycles of amplification followed by a 7-min extension at 72°C. Each cycle included denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 2 min. Amplified PCR products were visualized by gel electrophoresis of ten μl of product on a 1.6% agarose gel followed by ethidium bromide staining for 15 min. The specificities of the amplified bands were confirmed by their predicted product size based on a molecular weight standard. Positive (24-hr Con A stimulated equine PBMC) and negative (reaction master-mix only) controls were added to identify specific and non-specific amplification during PCR.

Real-time PCR utilizes the 5' to 3' endonuclease activity of AmpliTaq Gold™ DNA Polymerase to cleave a TaqMan® probe. During PCR, if the target molecules are present, the probe specifically anneals between the forward and reverse primer sites. The TaqMan® probe contains a reporter dye (6-carboxyfluoresceine) at the 5' end and a quencher dye (6-carboxytetrmethylrhodamine) at the 3' end. The un-reacted 3' nucleotide is blocked by phosphorylation, preventing elongation of the Taq DNA polymerase and dramatically reducing the flourescence of the reporter dye. The endonuclease activity at the 5' end of AmpliTaq Gold causes the probe to be cleaved allowing the separation of the reporter and quencher dyes resulting in an increased flourescence of the reporter dye. The accumulation of PCR products was detected by an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). The amount of cytokine mRNA in test samples was expressed as a fold difference compared to that seen in

equine PBMC after 24-hr Con A stimulation (positive control). The control sample provided a source of cDNA with an adequate expression of cytokine mRNA that could be used as a standard for each PCR reaction. Cytokine mRNA expression in the cDNA standard was used to compare to that seen in test samples and subsequently reported in fold-differences.

Real-time PCR analysis

All real-time PCR reactions were performed in special optical tubes that focus the flourescence signal in a 96-well format. PCR reactions were completed in an ABI PRISM 7700 Sequence Detector System (Perkin Elmer, Foster City, CA). Forward and reverse primers (900 nM/µl) for target cytokine (IL-2, INF-γ, IL-4, and IL-6) sequences and TaqMan® probes (250 nM/µl) (Applied Biosystems, Foster City, CA) were reacted with 12 µl of TaqMan Universal PCR Master Mix, 4.5 µl of DEPC treated water, and 2 µl of sample cDNA per tube. The internal probes were labeled at the 5' prime end with the reporter dye 6-carboxyfluoresceine, and at the 3' prime end with the quencher dye 6-carboxytetrmethylrhodamine. Fluorescence signals were generated during each PCR cycle by the 5'⇒ 3' endonuclease activity of AmpliTaq Gold™. Amplification was performed with initial incubation steps at 50°C for two min and 95°C for ten min followed by 40 cycles of 95°C for 15 min and 60°C for one min. All samples were assayed in triplicates and the mean values used for comparison. To account for variation in the amount and quality of starting material, all the results were normalized to G3PDH expression. Relative quantitation between samples was achieved by comparing their normalized threshold cycles (Ct). The Ct represents the PCR cycle at which an increase in reported flourescence above the threshold is detected. Samples without cDNA were included in the amplification reactions to determine background flourescence and check for contamination. cDNA from 24-hr Con A-stimulated equine PBMC was used as a positive control. The level of mRNA expression was reported as an "X"-fold difference to the calibrator. The samples used in the Realtime PCR assays

were those collected during animal studies to investigate the immune response of horses to vaccination and infection with EIV.

Results

Serology

The HI and SRH tests were used to screen horse sera for the presence of antibodies to various strains of EIV prior to their enrollment. The HI and SRH were also effective at detecting rises in serum antibody levels after vaccination and infection. Sera from day zero through 70 of the study were assayed by the HI and SRH method. While the HI assay was effective in establishing an antigen-specific antibody response in horse sera, the SRH test was found to be more sensitive than HI in detecting the appearance of circulating antibodies to EIV at an earlier time point after vaccination or infection. The sera from horses infected with the Kentucky-95 strain of EIV reacted with both Kentucky-95 and 94 antigen used the HI assay.

The ELISA has been used previously to detect isotype-specific immunoglobulin in equine sera and nasal secretions after vaccination and infection. In the present study, the ELISA provided a reliable method to measure serum IgGa and local IgA antibodies following vaccination and infection. The ELISA was used to screen horse sera for the presence of antibody prior to their enrollment. Hemagglutination and SRH tests were completed on the same sera to confirm the specificity of the ELISA. The sensitivity and specificity of the ELISA were further investigated and tests were conducted that provided a method to determine a "critical value" or cut-off value representing seroconversion. The OD at 490 nm was determined for each sample by an ELISA reader (Figures 2-1 and 2-2).

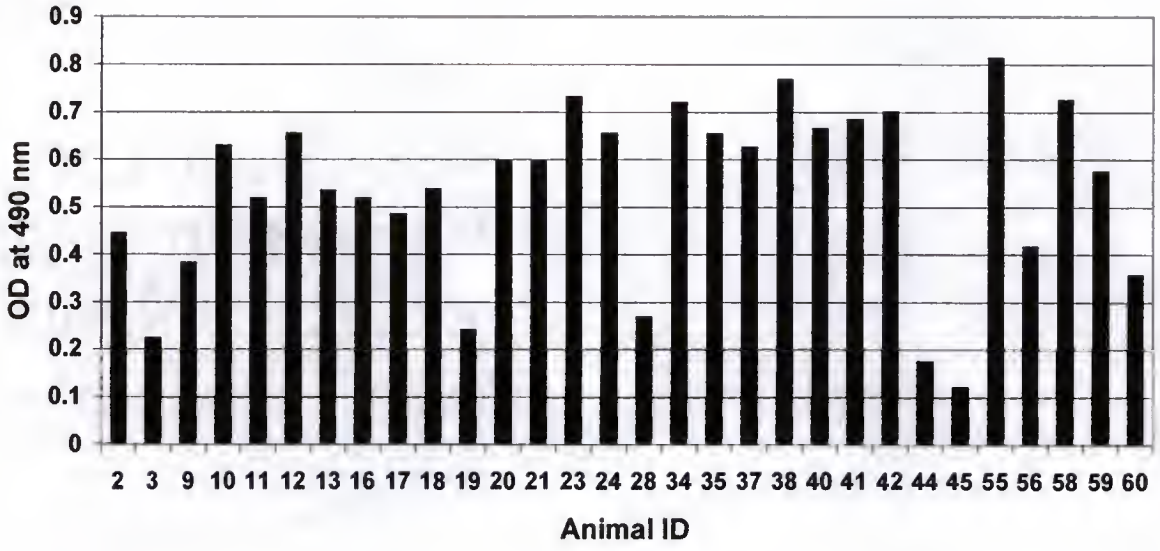


Figure 2-1 ELISA optical density readings from 30 horses that were confirmed as serum positive by hemagglutination inhibition and single radial hemolysis tests.

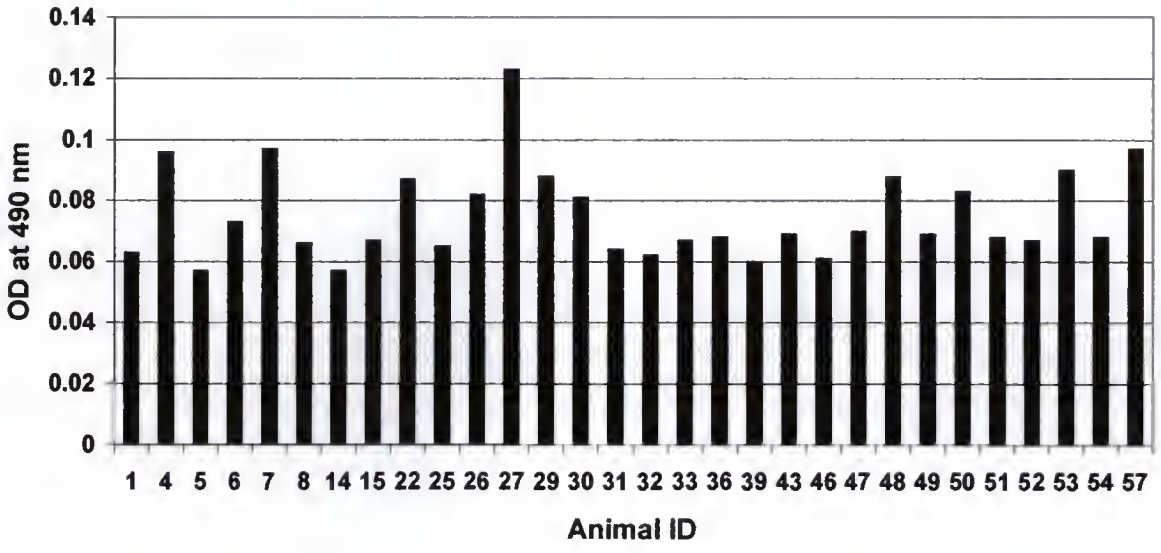


Figure 2-2. ELISA optical density readings from 30 horses that were confirmed as serum negative by hemagglutination inhibition and single radial hemolysis tests.
Note: Scale is 1/10 of previous graph.

Determination of the Critical or Cut off Value for the ELISA

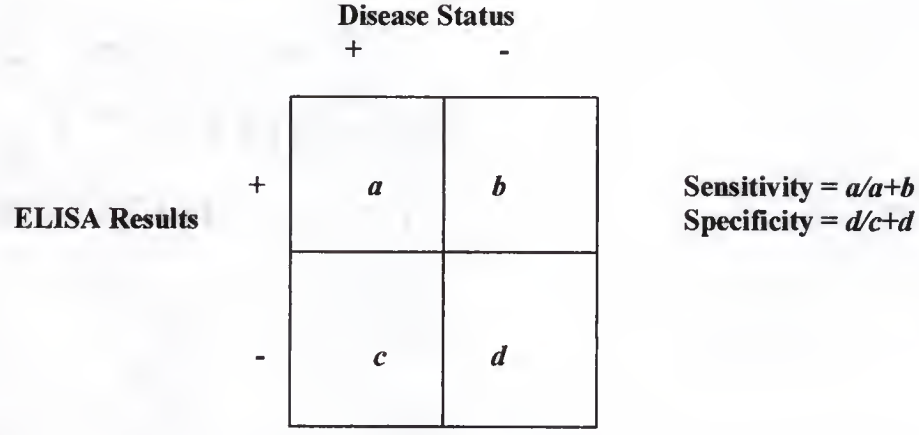


Figure 2-3. 2 x 2 tables used to calculate the sensitivity and specificity of The ELISA.

2 x 2 tables using data from Figures 2-1 and 2-2 were constructed to calculate the percent sensitivity and specificity at various OD readings (Figure 2-3). The ELISA was sensitive enough to detect antibodies in the sera of horses that were truly positive and distinguish from those that were truly negative. Zero of 30 sera in the seropositive group had an OD less than 0.1 and only two of 30 were less than 0.2. Further, only one serum sample from the seronegative group had an OD greater than 0.1. Table 2-1 summarizes the relative sensitivity and specificity at various critical values ranging from 0.05 to 1.0. Based on the data summary in Table 2-1, an OD reading of 0.1 correlated with the highest sensitivity and specificity.

Table 2-1 Critical (Cut-off) Values and the Associated Sensitivity and Specificity for the ELISA. Sensitivity and specificity at different cut-off values was determined using 2 x 2 tables described in figure 2-3.

Cut-off Values	% Sensitivity	% Specificity
0.05	100	0
0.06	100	7
0.07	100	57
0.08	100	63
0.09	100	87
0.1	100	97
0.2	93	100
0.3	83	100
0.4	77	100
0.5	67	100
0.6	43	100
0.7	20	100
0.8	3	100
0.9	0	100

Generating a "Critical-Value" Plot

A critical-value plot was constructed to show changes in sensitivity and specificity at various critical-values (Figure 2-4). As the critical value decreases, inclusion of a true-positive test-result produces an increase in the vertical trend reflecting higher sensitivity. Inclusion of a false-positive test-result produces a horizontal line. The point on the curve representing the

critical value (cut-off value) which lies closest to 100% for both sensitivity and specificity is indicated by the plot and was used to assess acute and post-inoculation sera. Based on these data, OD readings greater than 0.2 were considered to be representative of horses that were positive for EIV-specific antibody. `The ELISA provided a reliable quantitative method to detect serum IgGa antibody with minimal variability (standard deviation typically less than 0.1) between triplicate wells.

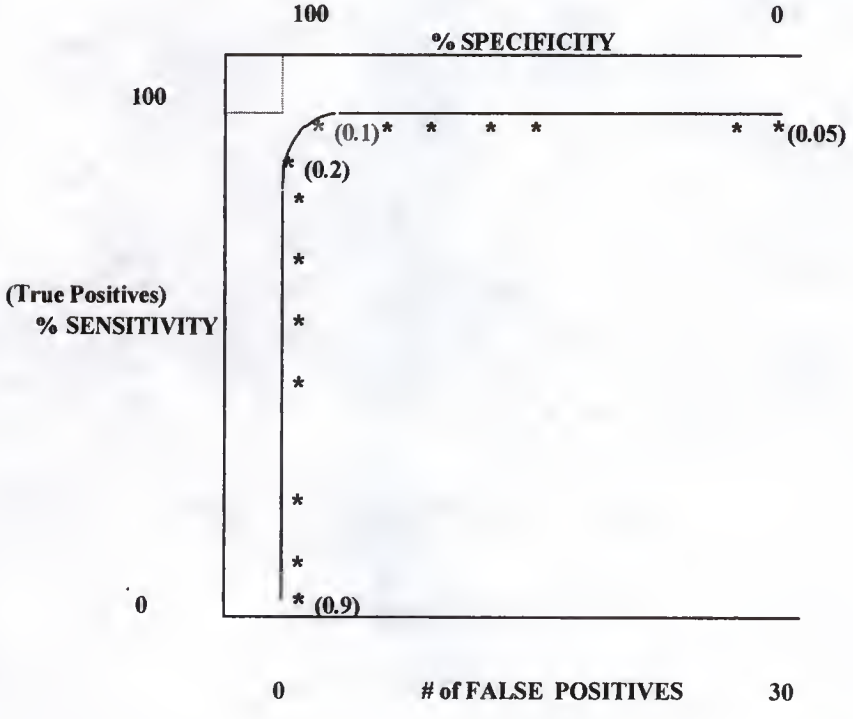


Figure 2-4. A Critical-Value plot of serum IgG antibody detected in seropositive and seronegative horses. Sensitivity and specificity for the ELISA are graphically represented by plotting the number of false positive (y axis) against the true positives (x axis).

Lymphocyte Proliferation Assay

Peripheral blood lymphocytes from horses in each group were used to determine the antigen-specific response to in-vitro stimulation with heat-inactivated A/equine/2/Kentucky/95

EIV in a 96-hr proliferation assay. Figure 2-5 indicates that the viability of cryopreserved equine PBMC averaged 89% upon thawing. Incubation of cultures for 18 hr to leach out cryopreservation reagents reduced the viability to 68%. Further, the process of freezing, thawing, the associated washes and centrifugation resulted in a 44% loss of cells. Con A was used to confirm the functional capacity of lymphocytes. Con A had a dose-dependent response in fresh and frozen equine PBMC. The optimum concentration of Con A was determined to be 5 μg/mL (Figure 2-6) and was used during the remainder of the study. A comparison of two assay methods to detect the blastogenic response of Con A-stimulated cells was performed. The blastogenic response, measured by a [^{3}H] Thymidine uptake assay, was comparable to that of the tetrazolium dye method (Figure 2-7). The stimulation index did not differ appreciably in either assay method. A SI greater than or equal to two was considered a positive antigen-specific proliferation response. All PBMC cultured during the blastogenesis assays were found to be immunologically functional by stimulation with either Con A or Pokeweed mitogen (data not shown).

Various preparations (heat-inactivated, UV-inactivated, and live) of EIV were used to determine if differences existed in the in-vitro response of equine PBMC. Based on the results represented in figure 2-8 and other experiments (data not shown), heat inactivated EIV was determined to induce a measurable blastogenic response in equine PBMC collected from horses following infection. Other assays using UV-inactivated virus demonstrated greater variability in the response (data not shown). No appreciable increase in the proliferation response was noted by using live virus compared to heat or UV-inactivated preparations as well.

Figure 2-9 shows a subtle change in the proliferation response from a range of infectious doses of EIV. Based on these results and other suggested protocols, an EID_{50} $10^{8.3}$ was used in subsequent assays. In the next experiment, the dose-dependent response of PBMC to hrIL-2 was assayed using EIV antigen and Con A mitogen. Figure 2-9 indicates a subtle difference in the Con A-stimulated response resulting from increasing concentrations of hrIL-2 in the CM.

However, a dose response was seen in PBMC stimulated with EIV when varying the concentration of hrIL-2. The stimulation index decreased when the hrIL-2 concentration was increased. In contrast, un-stimulated PBMC appeared to benefit from increased concentrations of hrIL-2. Based on these data, culture media was supplemented with 20 U/mL of hrIL-2 and used in subsequent proliferation assays.

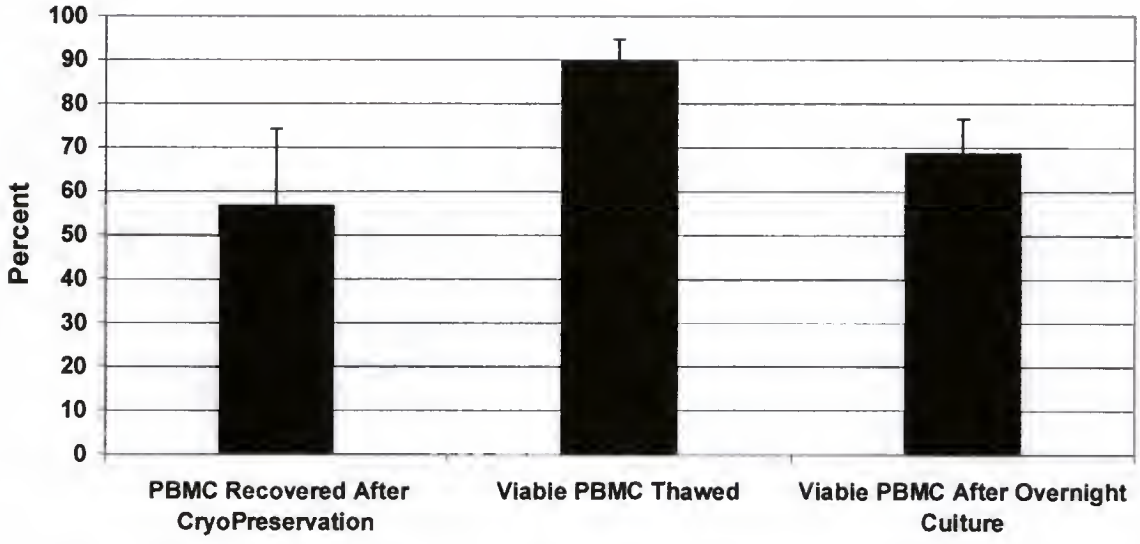


Figure 2-5. Changes in the total cell number recovered after thawing and viability after overnight incubation of cryopreserved equine peripheral blood mononuclear cells. The left hand-side bar reflects the total number of cells remaining after freezing, thawing, and washing. The center bar represents viability of cryopreserved equine peripheral blood mononuclear cells that were rapidly thawed in a 37° C water bath. Viability was determined by a trypan blue exclusion-dye method. The right hand-side bar shows a reduction in viability of frozen/thawed equine peripheral blood mononuclear cells after an 18-hr incubation at 37° C and 5% CO_2. Error bars represent ± SE of the mean.

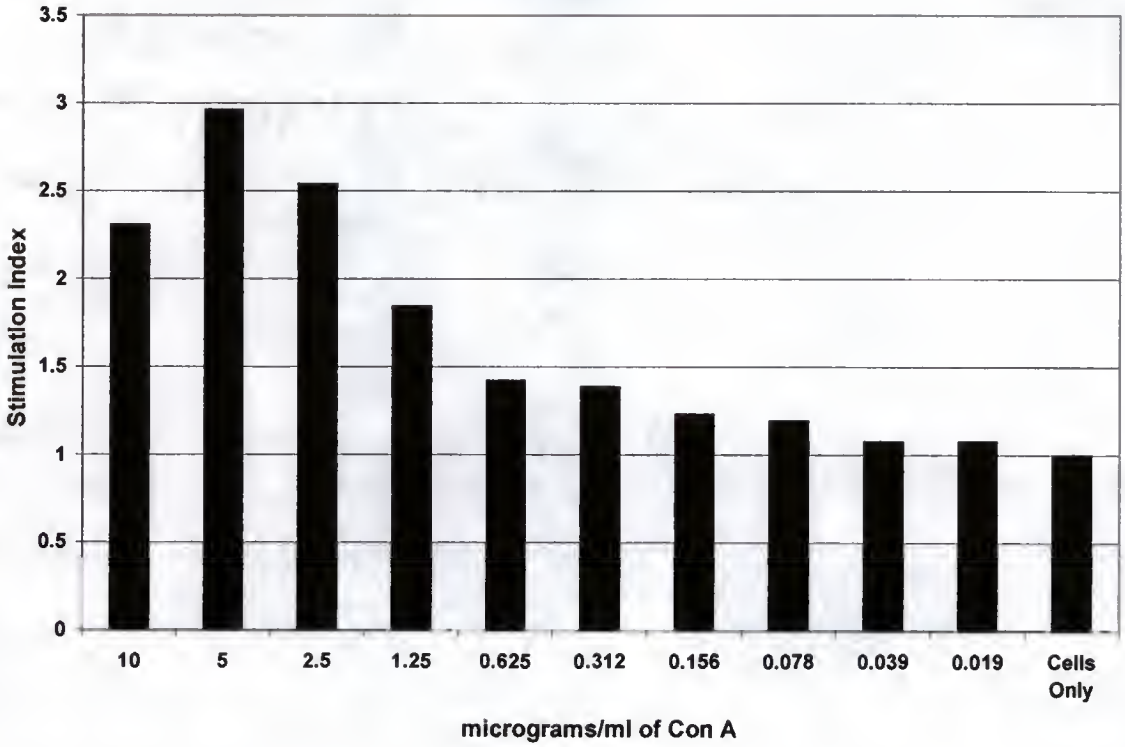


Figure 2-6. Dose-dependent response of equine peripheral blood mononuclear cells (1 x 10^5 cells/ml) to Concanavalin A during a 72-hr proliferation assay. Freshly isolated equine peripheral blood mononuclear cells were stimulated in vitro under culture conditions described in materials and methods. The mitogen-induced proliferative response was measured using a colormetric, tetrazolium dye assay method, also described in materials and methods. Data is representative of three separate experiments.

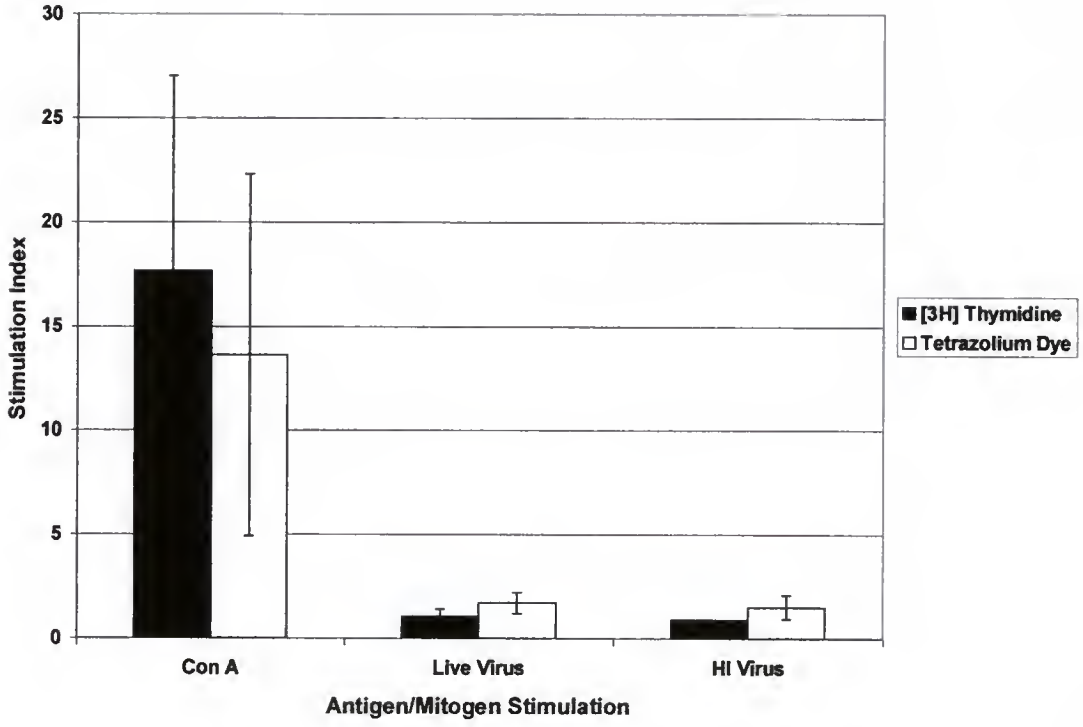


Figure 2-7. Comparison of [^{3}H] Thymidine and a tetrazolium dye colormetric assay methods to detect equine lymphocyte proliferation. Equine peripheral blood mononuclear cells were stimulated in vitro with Concanavalin A at 5 μg/mL for 72 and the proliferative response was then measured by both [^{3}H] thymidine incorporation and a tetrazolium dye colormetric assay. Data is representative of individual assays from three different horses.

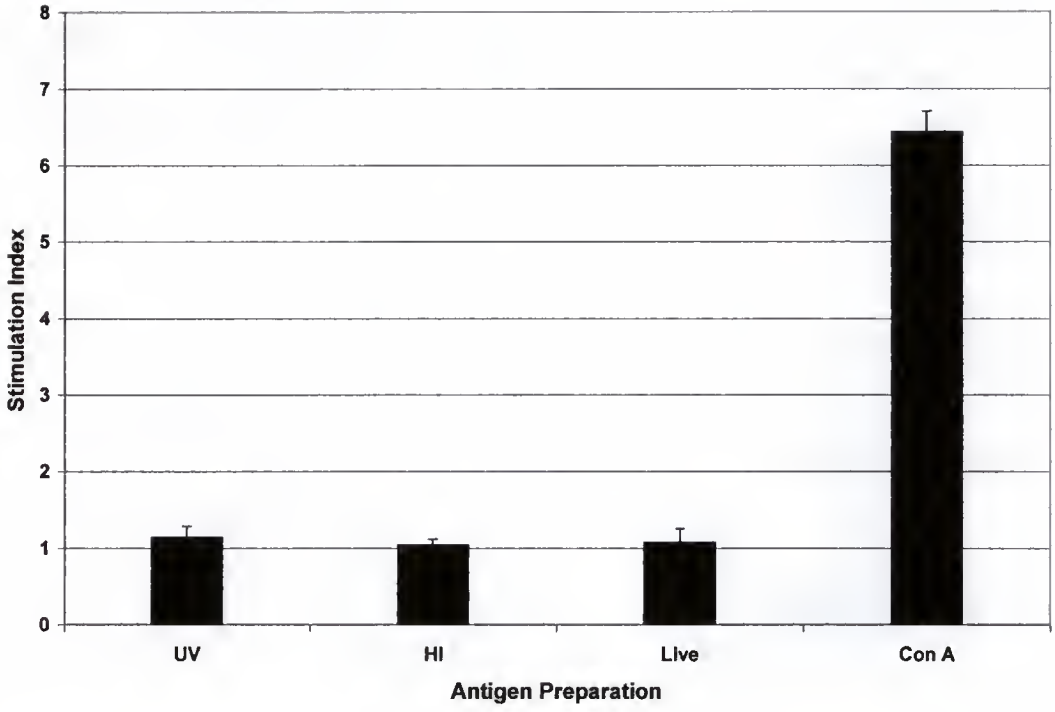


Figure 2-8. Comparison of various methods of antigen preparation (heat inactivation, UV inactivation, and live virus) for in-vitro stimulation of peripheral blood mononuclear cells with equine influenza virus. Equine influenza virus was inactivated by UV light using a technique described elsewhere {Rott & Cash 1994 732 /id}, or heat inactivated at 57° C for 30 min. Cells were stimulated with live virus at an infectious dose of $10^{8.3}$ EID_{50}. Concanavalin A was used at a concentration of 5 μg/mL. Cells were isolated from six horses, eight days after infection and were cryopreserved prior to the proliferation assay. Error bars represent ± SE of the mean.

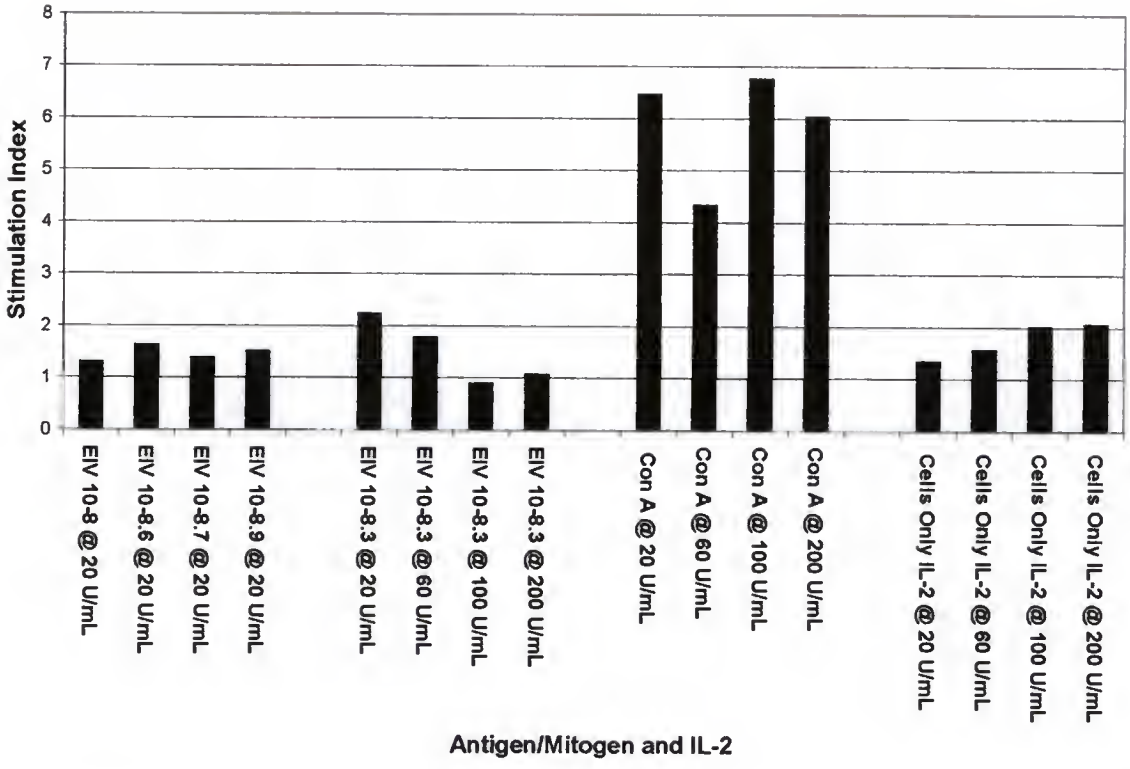


Figure 2-9. The in-vitro dose-dependent response of equine peripheral blood mononuclear cells to varying amounts of EIV and human recombinant IL-2 supplementation was determined in peripheral blood mononuclear cells isolated from horses 14 days after infection with EIV. The proliferative response of cells to varying amounts of equine influenza virus was determined with rhIL-2 supplemented to the culture medium as indicated. In a separate series of experiments, the dose of EIV was held constant and the effect of increasing amounts of rhIL-2 was determined. The response of equine peripheral blood mononuclear cells to increasing concentrations of rhIL-2 was then determined in both mitogen stimulated and un-stimulated cultures, as indicated.

Cytokine mRNA Determination in Cryopreserved Equine PBMC

Reverse transcription of RNA was followed by conventional PCR methods to confirm β-actin mRNA in PBMC stimulated in vitro with EIV for 72 hr. Figure 2-10 is representative of several experiments showing the relative expression of β-actin in cDNA samples using rt-PCR assays. After β-actin was confirmed in each sample, cDNA was analyzed for G3PDH and cytokine mRNA expression by Realtime PCR. The relative expression of G3PDH was found to be comparable to levels expressed in Con A-stimulated cells. Total cDNA was then analyzed for IL-2, INF-γ, IL-4, and IL-6 mRNA expression. Whereas levels of IL-2, INF-γ, and IL-6 were readily detected in most of the samples, IL-4 was expressed at very low levels. There were no clear patterns of cytokine expression in samples from vaccinated and EIV-infected horses. Furthermore, cytokine mRNA from in-vitro stimulated equine PBMC was highly variable and did not show a pattern of vaccine-induced expression (Figures 2-11 through 2-14).

It was speculated that a 72-hr stimulation time was too long to detect peak levels of cytokine mRNA expression in cultured PBMC. Therefore, a second experiment was conducted to determine the relative expression of IL-2 mRNA in PBMC from two vaccinated horses after an 8-hr stimulation. An increase was noted in both samples after an 8-hr in-vitro stimulation (Figures 2-15 and 2-16). As indicated in the graphs, the pattern of IL-2 expression was reversed in the 8-hr culture compared to that seen after 72-hr in-vitro stimulation. These data indicate that frozen PBMC from horses can be successfully assayed for cytokine expression using Realtime PCR methods. Based on these results, subsequent cytokine analysis was conducted in 8-hr in-vitro stimulation cultures.

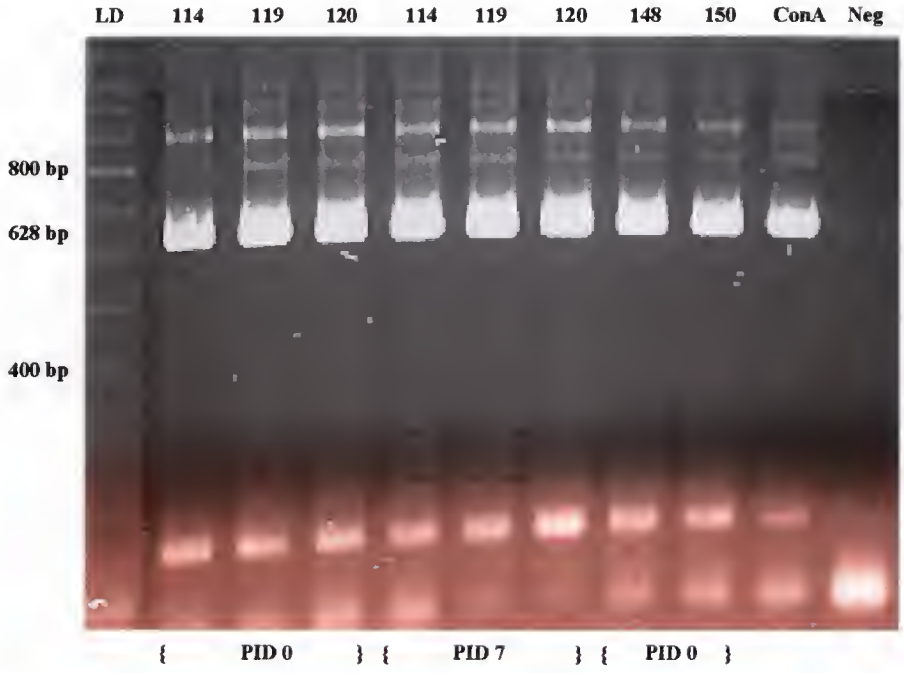


Figure 2-10. Amplified PCR products representing β actin (628 bp size fragment) mRNA expression visualized by gel electrophoresis and ethidium bromide staining. Equine peripheral blood mononuclear cells were stimulated in-vitro, total RNA was isolated, and cDNA was generated by reverse transcription. cDNA was confirmed by a conventional PCR assay using primers for β actin. These data are representative of mRNA isolated from in-vivo-primed cells collected at various time points (post inoculation days [PID] 0 and 7) after vaccination and infection with equine influenza virus.

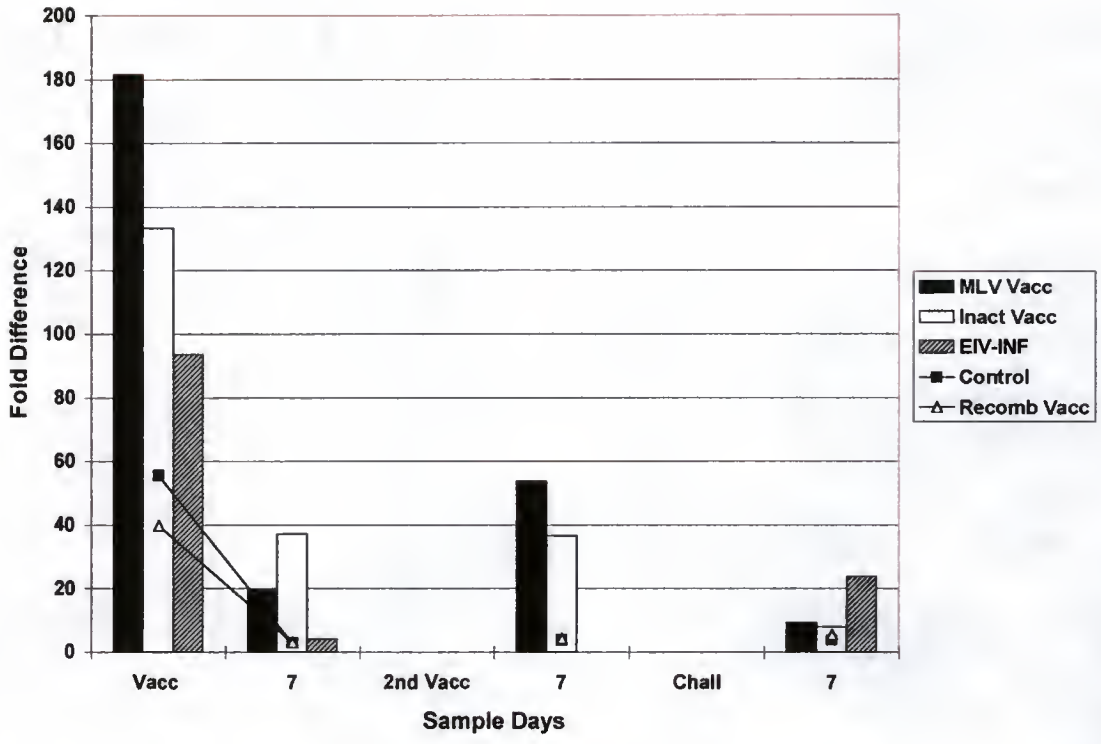


Figure 2-11. IL-2 mRNA expression in in-vivo primed peripheral blood mononuclear cells from vaccinated and infected horses during a 72-hr in-vitro stimulation with heat-inactivated equine influenza virus antigen. Equine peripheral blood mononuclear cells were stimulated in-vitro, total RNA was isolated, and cDNA was generated by reverse transcription. A Realtime PCR assay was conducted to show changes in mRNA cytokine expression at various times (day of vaccination [Vacc], 7 days after vaccination or challenge [Chall]) as indicated. Cytokine detection assays were not done on 2nd Vacc or Chall days. Changes are expressed as a x-fold difference above a Con A control. (Vaccines and EIV-INF groups n = 3; Control n = 1)

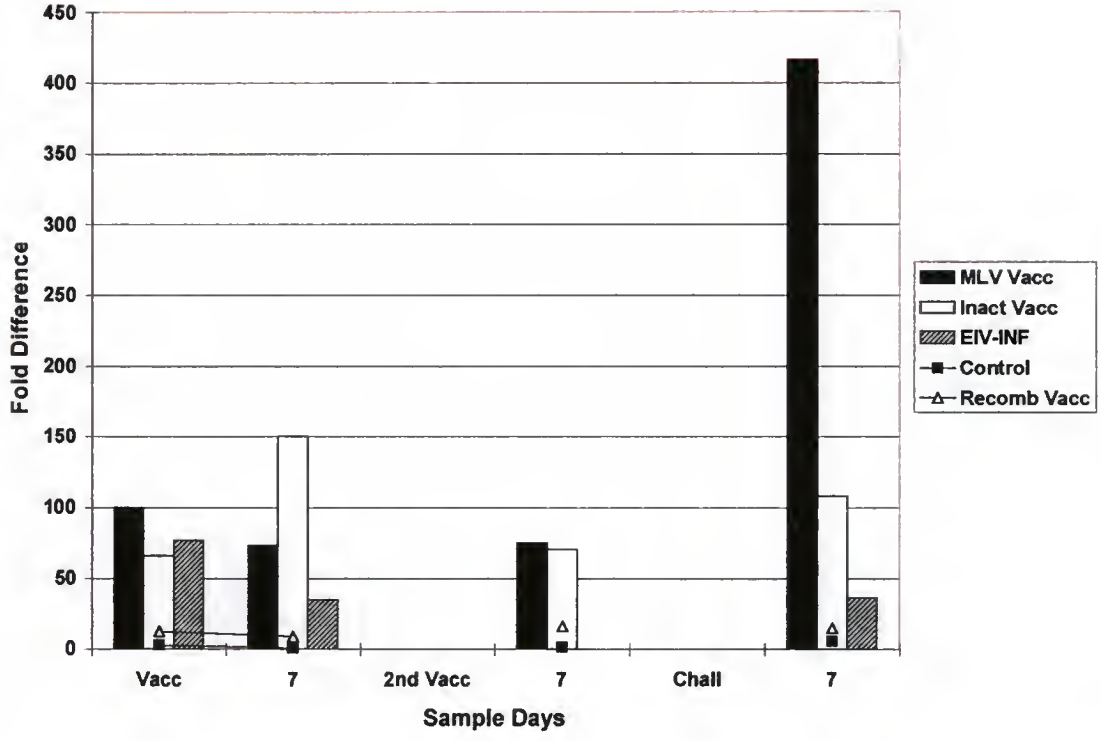


Figure 2-12. INF-γ mRNA expression in <u>in-vivo</u> primed peripheral blood mononuclear cells from vaccinated and infected horses during a 72-hr in-vitro stimulation with heat-inactivated equine influenza virus antigen. Equine peripheral blood mononuclear cells were stimulated <u>in-vitro</u>, total RNA was isolated, and cDNA was generated by reverse transcription. A Realtime PCR assay was conducted to show changes in mRNA cytokine expression at various times (day of vaccination [Vacc], 7 days after vaccination or challenge [Chall]) as indicated. Cytokine detection assays were not done on 2nd Vacc or Chall days. Changes are expressed as a x-fold difference above a Con A control. (Vaccines and EIV-INF groups n = 3; Control n = 1)

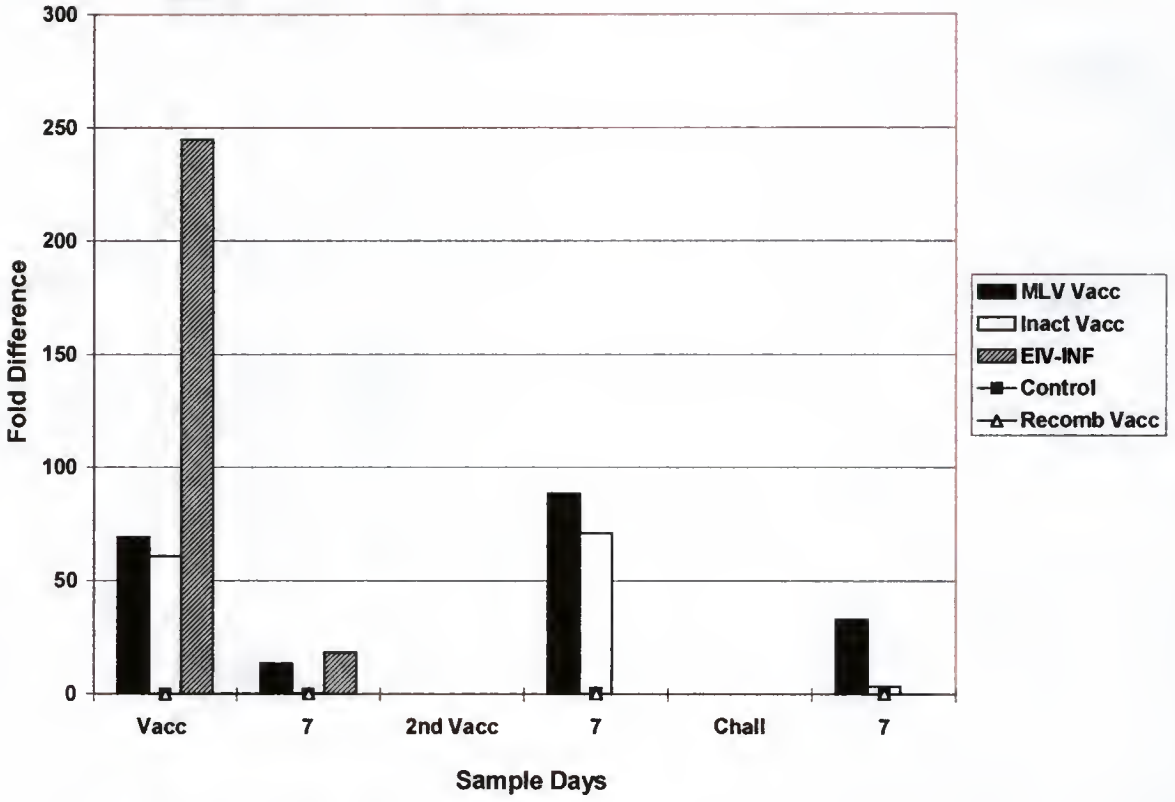


Figure 2-13. IL-4 mRNA expression in in-vivo primed peripheral blood mononuclear cells from vaccinated and infected horses during a 72-hr in-vitro stimulation with heat-inactivated equine influenza virus antigen. Equine peripheral blood mononuclear cells were stimulated in-vitro, total RNA was isolated, and cDNA was generated by reverse transcription. A Realtime PCR assay was conducted to show changes in mRNA cytokine expression at various times (day of vaccination [Vacc], 7 days after vaccination or challenge [Chall]) as indicated. Cytokine detection assays were not done on 2nd Vacc or Chall days. Changes are expressed as a x-fold difference above a Con A control. (Vaccines and EIV-INF groups n = 3; Control n = 1)

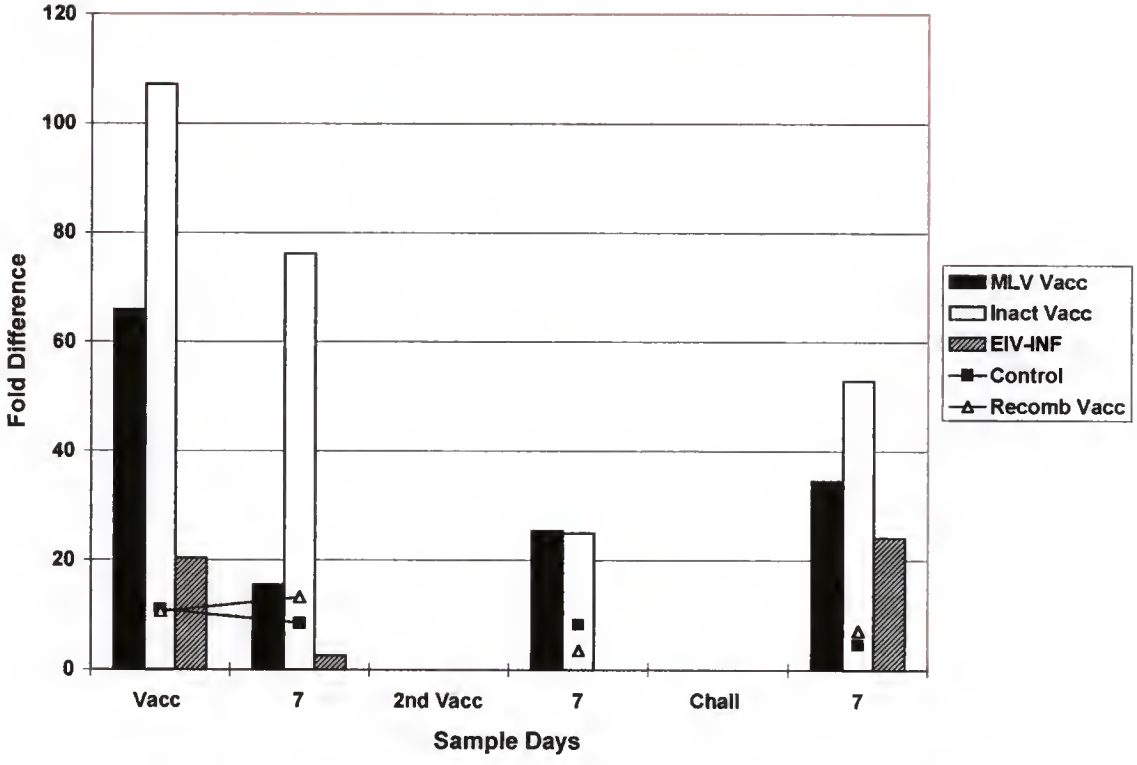


Figure 2-14. IL-6 mRNA expression in in-vivo primed peripheral blood mononuclear cells from vaccinated and infected horses during a 72-hr in-vitro stimulation with heat-inactivated equine influenza virus antigen. Equine peripheral blood mononuclear cells were stimulated in-vitro, total RNA was isolated, and cDNA was generated by reverse transcription. A Realtime PCR assay was conducted to show changes in mRNA cytokine expression at various times (day of vaccination [Vacc], 7 days after vaccination or challenge [Chall]) as indicated. Cytokine detection assays were not done on 2nd Vacc or Chall days. Changes are expressed as a x-fold difference above a Con A control. (Vaccines and EIV-INF groups n = 3; Control n = 1)

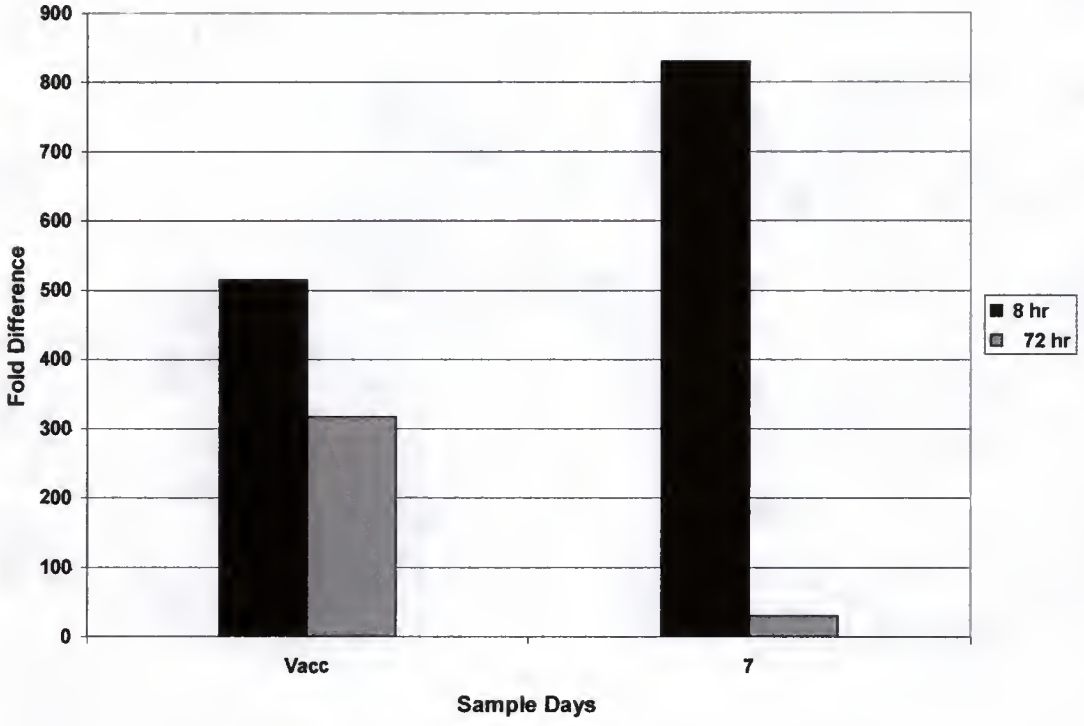


Figure 2-15. IL-2 mRNA expression in peripheral blood mononuclear cells from horse # 146 in the modified-live vaccine group. Equine peripheral blood mononuclear cells underwent in vitro stimulation for 72 or 8 hr; total RNA was isolated, and cDNA was generated by reverse transcription. A Realtime PCR assay was conducted to detect changes in mRNA cytokine expression on the day of and 7 days following vaccination (Vacc) as indicated. Changes are expressed as a x-fold difference above a Con A control.

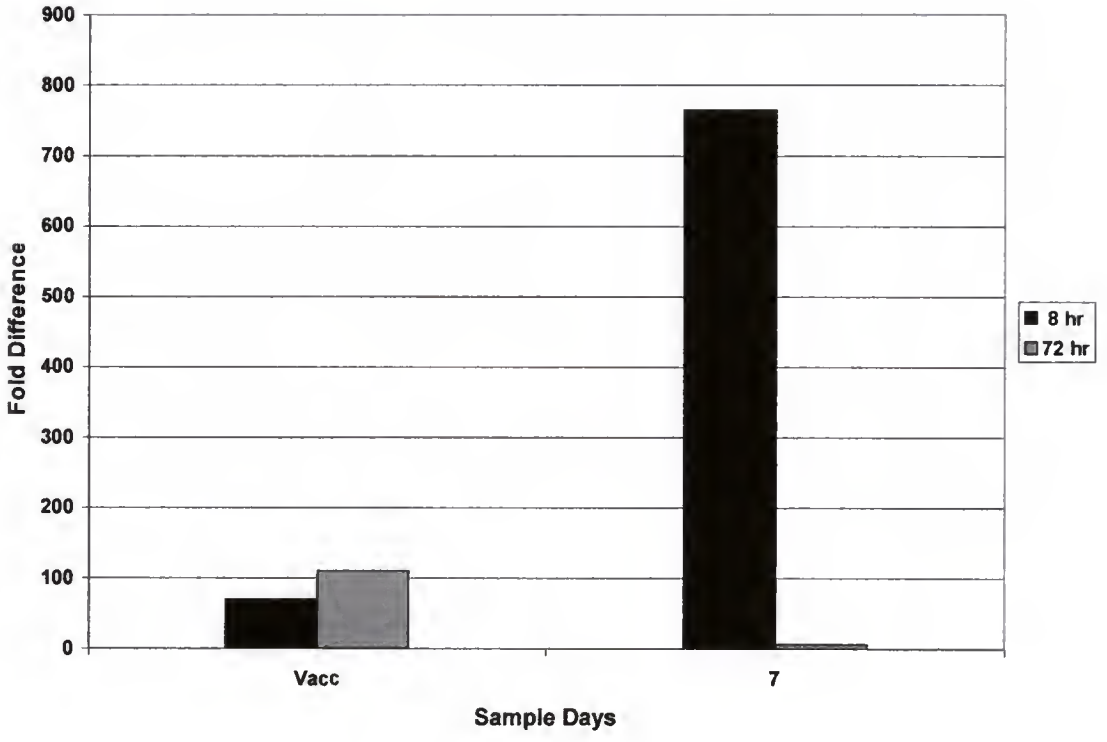


Figure 2-16. IL-2 mRNA expression in peripheral blood mononuclear cells from horse # 149 in the inactivated virus vaccine group. Equine peripheral blood mononuclear cells underwent in vitro stimulation for 72 or 8 hr; total RNA was isolated, and cDNA was generated by reverse transcription. A Realtime PCR assay was conducted to detect changes in mRNA cytokine expression on the day of and 7 days following vaccination (Vacc) as indicated. Changes are expressed as a x-fold difference above a Con A control.

Discussion

Historically, HI, SRH, and virus neutralization assays have been the gold-standard for measuring sero-conversion to vaccination or infection with EIV(Wilson, 1993) (Mumford, 1991). These assay methods have been shown to correlate closely with virus neutralizing antibodies (Morley, Hanson, Bogdan, Townsend, Appleton, & Haines, 1995). The HI assay is the most widely used serological indicator of immunization against or infection with influenza in humans, mice, and horses. It is relatively inexpensive, rapid, and can differentiate between types 1 and 2 of equine influenza (Wilson, 1993). The specificity of the HI test is based on the principle that it detects antibodies to the HA surface protein which is subject to antigenic drift . However, in our hands this assay was not as sensitive as either SRH or ELISA techniques to detect seroconversion at an early time point after exposure to EIV.

Because a four-fold increase in the HI antibody titer is considered diagnostic of exposure to influenza antigen, detection of a sub-clinical infection is possible. However, variability between laboratories, assay technicians, and individual assays has been reported (Mumford, 1991). Some of the variability can be explained by the different methods used to disrupt the virus prior to its use as antigen. Furthermore, one study showed that antigen from influenza grown in cell culture (Madin-Darby canine kidney cells) is more sensitive to antibody than that derived from virus grown in chicken eggs (Cook, Mumford et al., 1988).

The SRH assay is widely accepted as a sensitive and reproducible indicator of sero-conversion and studies have shown that SRH titers ranging from 75 to 150 mm^2 correlate with protection against infection with EIV (Hannant, Mumford, & Jessett, 1988;Mumford, Wood, Scott, Folkers, & Schild, 1983;Mumford, Wood, Folkers, & Schild, 1988;Mumford, Jessett, Dunleavy, Wood, Hannant, Sundquist, & Cook, 1994a;Mumford, Wilson, Hannant, & Jessett, 1994;Townsend, Morley et al., 1999;Wood, Mumford, Folkers, Scott, & Schild, 1983). The

increased sensitivity to detect circulating antibodies to influenza by SRH assay over that of HI has been reported elsewhere (Ennis, F.A. et al., 1977).

In previous studies, detection of serum antibody to influenza vaccination and infection was restricted by assay methods that had limited sensitivity, specificity or provided results that were variable. In this study, ELISA techniques provided both a sensitive and specific assay to detect serum and nasal influenza-specific antibodies. Sensitivity and specificity are two indicators of the amount of validity of a diagnostic test. Sensitivity can be calculated from the relation *(a/a+b)* in a two by two table (Figure 2-4) and is described as the probability of having a positive test result from an animal that is truly diseased where "disease" can mean sub-clinical disease and or infection. Likewise, specificity is a measure of the probability of a negative test result for a sample from a truly negative animal *(d/c+d)*. By increasing the sensitivity of the test, the number of cases that have had exposure and are misdiagnosed as negative by the test (false negatives) will decrease. Appropriately so, a highly specific test will report a small number of positives in the absence of exposure. These are referred to as false positives. Because there will be a range of values for both diseased and non-diseased animals (low and high values in each category), there will be some overlap which results in an inverse relationship between the sensitivity and specificity of the ELISA measuring antibody titers. This relationship can be adjusted based on a selected critical value (cut off point). Therefore, the critical value will delineate those animals considered positive or negative for sero-conversion. Horses exposed to EIV with OD above the critical value are considered positive and are represented by *a* in the two by two table. Those exposed and having values below the critical value are represented by *c* and are false negatives. Horses that have not been vaccinated or experimentally infected and have values above or below the critical value are represented by *b* and *d* respectively.

The ELISA method was able to correlate an OD reading or "critical-value" to seropositive or seronegative status in horse sera. True positive or negative horses (based on known history and the determination of influenza-specific antibody by HI and SRH) were detected by the

ELISA with 100% and 97% sensitivity and specificity respectively. This type of analysis provided the basis to establish a cut-off value (OD value) of 0.1, reflecting the point where horses were considered to have seroconverted. Based on these parameters, this assay was further used in the current study to determine the serum and local antibody response post-vaccination or infection.

Due to varying proliferation assay methodologies, preliminary assays were conducted to optimize culture conditions for this study. Antigen preparation and the duration of incubation were optimized to obtain a measurable response in PBMC from horses prior to and after challenge infection. In some cases, samples were collected from horses used from ongoing investigations within our laboratory not directly related to the present study.

To confirm the ability of lymphocytes to respond to mitogenic stimulation, cultures containing Con A were included in the proliferation assays. Depending on the stimulus, the proliferation response can involve either T or B cells or both. For example, during mitogen-induced proliferation, phytohemagglutinin (PHA), and Con A preferentially induce T cells while PWM will result in both a T and B-cell response (Fletcher, Klimas, Morgan, & Gjerset, 1992). For antigenic peptides derived from endogenous or viral protein, class I MHC molecules are involved (Neefjes & Momburg, 1993;Rammensee, Falk et al., 1993). Extracellular antigens are processed via endosomal compartments, presented by MHC-II molecules, and activate $CD4^+$ T cells (Roitt, 1997).

One published study reports the dose-dependent response of equine PBMC to Con A at an optimum concentration of 20 μg/mL during a 4-dy incubation (Truax, Powell et al., 1990). In contrast, the present study demonstrated unfractionated equine PBMC had an optimum four-dy proliferation response to Con A at 5 μg/mL. Further, the addition of hrIL-2 at 200 U/mL did not show a significant increase over that seen with 20 U/mL during Con A stimulation. Others have reported lymphocyte blastogenesis in response to increased concentrations of hrIL-2 during

mitogen stimulation (Hammond, Issel et al., 1998) and the maintenance of long-term cultures of hrIL-2-dependent PBMC (Stott & Osburn, 1988). However, as might be expected with all non-human T lymphocytes, the response of equine PMBC to human IL-2 is less than would be seen by the addition of a species-specific IL-2 (Fenwick, Schore et al., 1988).

The number of animal studies that have included the use of cryopreserved PBMC is limited. Cryopreserved PBMC have been used in histocompatibility and mitogen-response experiments in sheep (Stear, Allen et al., 1982), cattle (Kleinschuster, VanKampen et al., 1979) and in horses (Truax, Powell, Montelaro, Issel, & Newman, 1990). In contrast, the mitogen-induced response of human lymphocytes after cryopreservation has been well characterized (see review) (Fletcher, Klimas, Morgan, & Gjerset, 1992). Furthermore, the successful use of human mononuclear cells after cryopreservation has been demonstrated (Allsopp, Nicholls et al., 1998;Lamb, Jr., Willoughby et al., 1995). One study reported that frozen/thawed human lymphocytes not only recover and respond to plant mitogens but showed a reduction in the variability associated with proliferation assay techniques (Sears & Rosenberg, 1977). Cryopreserved equine PBMCs have been used in separate studies to determine the CTL activity against equine herpes-virus (EHV) (Allen, Yeargan et al., 1995;O'Neill, Kydd et al., 1999). Consistent with other reports, where cryopreserved cells were 95% viable (Truax, Powell, Montelaro, Issel, & Newman, 1990), equine PBMC used in the present study were 89% viable immediately following a rapid thaw. The percentage of viable cells fell to 68% after 18-hr incubation in culture media however. The cause for the loss of cell viability may have been due to the lack of cytokines. However, the exact cause of cell death was not investigated. Cells that were viable after over-night incubation, were consistently capable of responding to Con A , Pokeweed mitogen and minimally to EIV antigen.

The proliferative response was measured by a colormetric dye method in place of a [^{3}H] thymidine uptake assay. Other studies have reported on the ability of a tetrazolium-salt-based dye to detect the proliferation response in cultured cells (Behl, Davis, Lesley, & Schubert,

1994;Lappalainen, Jaaskelainen, Syrjanen, Urtti, & Syrjanen, 1994;Wong & Goeddel, 1994). A comparison of the colormetric and the [^{3}H] thymidine uptake assays has been made elsewhere (Kitamura, Tange et al., 1989) and in our laboratory for their ability to detect a proliferative response. Assays conducted in the present study did not indicate that the [H^3] thymidine uptake method was appreciably more sensitive than the colormetric dye method. Furthermore, the colormetric method eliminated the need for specialized training and equipment associated with radioisotope use.

In-vitro exposure to influenza antigen will induce T lymphocyte proliferation (Chow, Beutner et al., 1979). However, these observations are based on the response of PBMC to inactivated preparations of virus (Chow, Beutner, & Ogra, 1979;Lazar & Wright, 1980). While one report demonstrates a depressed mitogen response by influenza-infected human lymphocytes, the proliferation response to the viral infection remained intact (Roberts, Jr. & Nichols, 1989). Furthermore, human lymphocytes, infected with influenza, can stimulate autologous cells in short-term cultures (Thompson, Lewis et al., 1973). Studies describing the use of inactivated stimulator-cells have been conducted as well (Moreno & Lipsky, 1986). Therefore, in the present study, a comparison between the use of paraformaldehyde-fixed, UV inactivated (Roberts, Jr. & Nichols, 1989) autologous antigen-presenting cells, or the addition of heat inactivated virus to PBMC cultures was investigated. The use of fixed EIV-infected cells proved to be complicated with no demonstrable increase in the proliferation response between cultures when compared to other methods, including heat or UV-inactivated antigen preparations or live virus. In some experiments, a higher percentage of cell death occurred in cultures containing UV-inactivated or live virus (data not shown). In addition, a comparison was made to determine the optimal incubation time. Others have described three (Wiley & Skehel, 1977) and four-dy (Ellis, Bogdan, & Kanara, 1995;Hammond, Cook, Lichtenstein, Issel, & Montelaro, 1997) incubation periods for proliferation assays. Based on observations made in the present study, heat-

inactivated virus was found to have a measurable blastogenic effect on PBMC and was used to determine the antigen-specific response in 96-hr proliferation assays.

We used real-time quantitative rt-PCR to demonstrate that in-vivo-primed PBMC isolated from whole blood, could be frozen, thawed, stimulated in vitro, and assayed for cytokine mRNA expression. Beta actin expression was confirmed from cDNA after reverse transcription of mRNA by conventional PCR and gel electrophoresis. Once cDNA was determined for each sample, real-time PCR assays were conducted to detect glyceraldehyde 3-phosphate dehydrogenase (G3PDH) and target cytokines.

In the present study, mRNA cytokine production after in-vitro stimulation with inactivated EIV was investigated. PBMC from horses that had either been infected or immunized with one of three different vaccine preparations were stimulated in vitro for three days and assayed for cytokine mRNA expression. While one study shows expression of INF-γ up to 48 hrs after in culture, it also demonstrated an early peak-expression of IL-2 and IL-4 with a subsequent decline after four hr post-stimulation (Kruse, Moriabadi, Toyka, & Rieckmann, 2001). The importance of the stimulation-time was demonstrated in the present study. Interferon-γ levels increased in PBMC from horses on seven day after the first and second vaccination and infection. In contrast, there was a dramatic drop in IL-2 levels on day seven after the initial vaccination or infection. A similar trend was noted in IL-4 and IL-6. While it is conceivable that IL-4 levels may be lower than IL-2 and INF-γ in response to infection, a similar pattern of IL-4 expression was seen in the inactivated-virus vaccine group and negative control. Based on studies in mice, IL-6 may have been expected to rise after either vaccination or infection (Matsuo, Iwasaki et al., 2000). A possible explanation for the highly variable cytokine mRNA expression in the present study is that the stimulation time of PBMC was too long (72 hrs), and peak expression of INF-γ, IL-2, IL-4, and IL-6 was within the first four to 48 hours after antigen stimulation. These data have not been published for horses. Therefore, a pilot study to investigate the kinetics of cytokine

expression in PBMC at various times during a 48-hr in vitro stimulation with Con A would have been appropriate. To make this comparison, however, would have required additional lengthy animal studies. Speculation as to why mRNA levels at day zero were many-fold higher than at dy-7 should include consideration of the molecular events regulating the memory cell phenotype. Based on previous studies already mentioned, an increase in IL-2 mRNA cytokine expression was expected day seven after inoculation with either live virus or an MLV vaccine. In mice, differential cytokine expression can be detected within hours of stimulation. However, the data show a dramatic decline in all groups on day seven compared to values on day zero. The dramatic decrease in cytokine levels on day seven may be associated with the in-vitro culture time. By the third day of incubation, the cellular processes responsible for gene expression were depleted in PBMC responding in an antigen-specific (primed) manner but not those that had no previous exposure to EIV antigen. This idea is supported by the fact that PBMC on day zero may have not undergone an increased upregulation of cellular mechanisms (gene expression) in response to antigen stimulation. Interferon-gamma and IL-2 mRNA from un-stimulated mouse lymphocytes were shown to reach maximum expression on day seven after influenza infection (Matsuo, Iwasaki, Asanuma, Yoshikawa, Chen, Tsujimoto, Kurata, & Tamura, 2000) (Mosmann & Coffman, 1989). Also, murine lymphocytes have been shown to reach maximum expression of INF-γ and IL-2 in day seven post-infection samples after 24-hr in-vitro stimulation (Asanuma, Aizawa, Kurata, & Tamura, 1998). Furthermore, expression of IL-4 and IL-6 has been shown to peak in nasal associated lymphocytes of mice seven days after vaccination and infection with influenza, respectively (Matsuo, Iwasaki, Asanuma, Yoshikawa, Chen, Tsujimoto, Kurata, & Tamura, 2000). Again, in the Matsuo study, no in-vitro stimulation was used. In other studies, cytokines have been measured in the supernatants of human macrophage and lymphocyte cultures after infection with influenza. In humans, INF-γ, IL-2, IL-4, and IL-6 activity was quantified in the supernatants of PBMC post-vaccination by ELISA methods (McElhaney, Upshaw et al.,

1998). In another study, peak protein levels of INF-γ have been measured in the supernatants of mouse macrophage cultures seven days after infection (Monteiro, Harvey et al., 1998).

The hypothesis (relative to the current study) that peak cytokine mRNA expression was missed in vitro and the related cellular mechanisms were exhausted in cells primed in vivo but not un-primed cells by the third day in culture is further supported by Figure 2-15. To investigate the earlier expression of IL-2 mRNA, PBMC from two horses (# 146 in the MLV vaccine group and # 149 in the inactivated vaccine group) were stimulated for 8 hr and assayed for cytokine expression as described above. Day zero IL-2 levels induced by stimulation for eight hours, were within only a 1.6 fold difference of those resulting from a 72-hr stimulation. However, a rise in IL-2 mRNA expression in dy-7 samples was noted in the 8-hr stimulation cultures. Furthermore, overall levels of IL-2 were higher in the 8-hr versus the 72-hr cultures. Again, this is most likely due to the fact that the PCR assay is able to detect relative mRNA expression at a particular point in time and is a measure of the in-vivo-primed cell's ability to respond in an antigen-specific manner. Based on this information, additional tests are ongoing to determine the differential cytokine mRNA expression in PBMC using an 8-hr in-vitro stimulation assay. It will be helpful to determine the cytokine response in addition to other measures of immunity in order to fully characterize the relationships between each.

In summary, it appears that the standardized assay methods described above can be used to characterize the immune mechanisms in horses. The ELISA could be used to detect small increases in serum and local antibody with a sensitivity and specificity of 100% and 97%, respectively. Furthermore, use of rayon-tipped proctoscopic swabs proved to be an easy and efficient method to collect nasal secretions from horses. Lastly, the above defined culture conditions are appropriate to assay cryopreserved equine PBMC in 98-hr lymphocyte proliferation assays and cytokine mRNA expression analysis.

CHAPTER 3
STANDARDIZATION OF ANIMAL INFECTION AND SAMPLING METHODS TO STUDY THE IMMUNE RESPONSE OF HORSES DURING EQUINE INFLUENZA INFECTION

Introduction

One of the difficulties in the field of equine influenza is the diversity of techniques associated with challenge-infection models. Specifically, various methods have been used to challenge/inoculate experimental animals with EIV during vaccine efficacy testing. Methods of inoculation have included intranasal instillation (Mumford, Wood, Scott, Folkers, & Schild, 1983;Mumford, Wood, Folkers, & Schild, 1988) and aerosolization of live virus into an enclosed stall by use of a nebulizer (Mumford, Wilson, Hannant, & Jessett, 1994). Nebulizers have been the more recent choice because they aerosolize virus in droplets with a diameter of less than 5μ that reaches the upper respiratory tract (Hannant, unpublished data) and more closely mimic the "natural" route of infection. Aerosolization of virus avoids concentrating the inoculum at the sample site as well. Depending upon the strain and titer, clinical signs resulting from experimental infection can range from mild to severe (Mumford, Hannant et al., 1990). During natural infection, it is speculated that fewer virus particles are needed to disseminate the disease. It is rare to isolate a titer of more than 10^3 EID_{50}/mL from horses naturally infected with EIV (Mumford, Jessett et al., 1994b). Furthermore, while nebulisation with as little as 10^2 EID_{50}/mL is capable of causing an infection, a dose of 10^6 EID_{50}/mL is required to cause severe clinical disease (Mumford, Hannant, & Jessett, 1990). In the present study, a variation in previous techniques was investigated. Infection of individual horses was performed by aerosolization of virus.

Local antibody production is a significant component of protection against infection with influenza. Various other methods to collect nasal secretions include nasal washes (Boyce, Gruber, Sannells, & et.al, 2000;Tamura, Funato, Hirabayashi, Kikuta, Suzuki, Nagamine, Aizawa, Nakagawa, & Kurata, 1990;Tamura, Funato, Hirabayashi, Suzuki, Nagamine, Aizawa, & Kurata, 1991) or in the case of horses, the insertion of tampons into the nasal passage for 15 min (Lunn, Soboll, Schram, Quass, McGregor, Drape, Macklin, McCabe, Swain, & Olsen, 1999;Nelson, Schram, McGregor, Sheoran, Olsen, & Lunn, 1998). The use of rayon-tipped proctoscopic swabs to collect nasal secretions from the nostrils of horses was investigated. Further, the amount of fluid and antibody retained by the swabs was determined prior to their use in animal studies.

Materials and Methods

Horses

Belgian and Percheron draught horses of both sex and approximately 12 months-old were purchased from a farm in Manitoba, Canada. Prior to their purchase, horses were bled and sera were screened for antibodies to EIV. Horses that were identified as being sero-negative to EIV (by HI and SRH) were purchased and transported to the UF equine research center in Ocala, Florida. To avoid the potential of exposure to viral pathogens typically found in stock-yards, the draught horses were watered and fed in the trailer. An HI and an ELISA were performed on each animal again prior to enrolling them in the study. All studies were approved by the UF Institutional Animal Care and Use Committee (IACUC).

Infection Strains of Equine Influenza Virus

The EIV strain A/equine/2/Kentucky/95 was obtained from Dr. Tom Chambers, from the University of Kentucky at the second egg-passage. The virus was passaged in eggs a further time and frozen at –70° C until used. The virus was tested for influenza by hemagglutination and titrated in 10-day-old embryonated chick eggs.

Infection Model Validation Study

A validation study was conducted to establish a reliable method to infect horses with EIV that would result in seroconversion and clinical disease. In this study, clinical disease was defined as an increase in temperature over 101.9° F (38.7° C), appearance of ocular or nasal discharge, anorexia, coughing, and lethargy. Two groups of randomly allocated horses, six in each with two negative controls, were infected with a "high dose" (10^8 EID_{50}/animal) or a "low dose" (10^5 EID_{50}/animal) of A/equine/2/Kentucky/95 EIV. Horses were housed in individual stalls within Progress Center's large-animal (biosafety level 2 facility) research barn during the infection procedure and up to ten days afterward. Individual horses were infected by aerosolization of 5-mL suspension of virus-infected allantioc fluid diluted in sterile PBS. An equine AeorMask™ (Turdell Medical, South London, Ontario, Canada) connected to a Devilbiss (Ultra-Neb 99 Ultrasonic, Model 009HD) nebulizer was placed over the nose to deliver aerosolized virus (Figure 3-1). Horses were monitored daily for the appearance of clinical disease and bled once a week for two weeks after infection. A clinical scoring system was used to provide a subjective measure of clinical disease (Table 3-1). Clinical score observations were made by individuals that were un-blinded to the treatment groups.

Table 3-1. Scoring system associated with clinical signs observed in horses following infection with equine influenza virus

Parameter	Observation	Assessed Value
Rectal Temperature in Degrees F	**98.5-101.9**	**0**
	102.0-103.0	**1**
	103.1-104.0	**2**
Nasal Discharge	**Yes**	**1**
	No	**0**
Anorexia	**Yes**	**2**
	No	**0**
Coughing	**Yes**	**1**
	No	**0**
Lethargy	**Yes**	**1**
	No	**0**

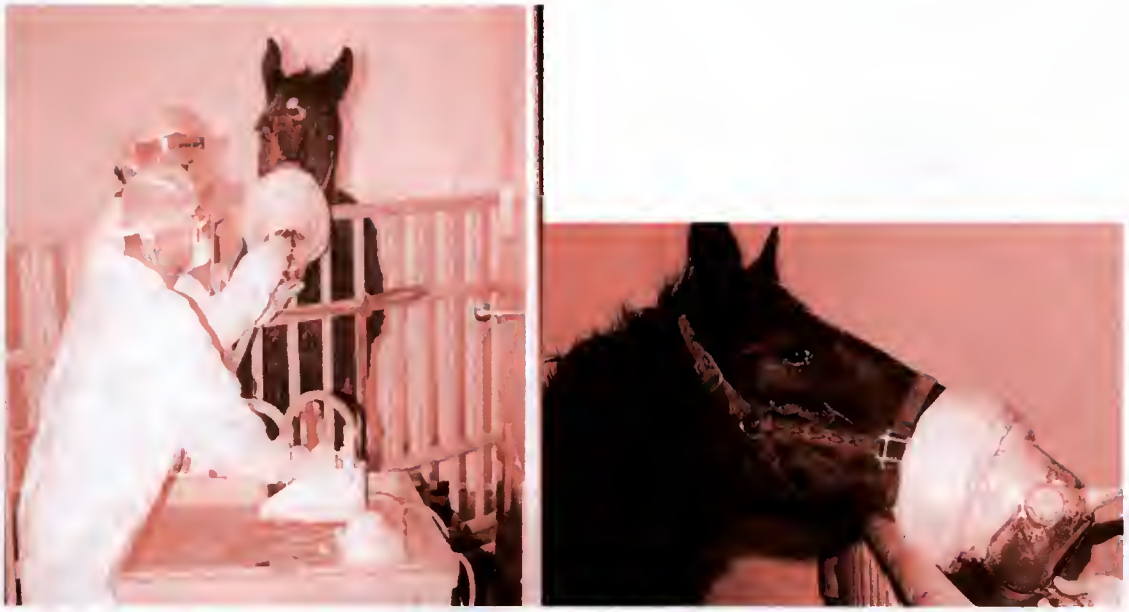


Figure 3-1. Infection of horses with equine influenza virus within isolation stalls using a nebulizer connected to an equine AeroMask™

Nasal Secretion Sampling

Horses were placed in stocks with their heads restrained allowing minimal movement. A 16-in rayon-tipped proctoscopic swab (Quality Medical Products, Guilton, MN) was inserted 15-cm-deep into the ventral meatus of each nostril where it remained in place for five min (Figure 3-2). Care was taken to avoid trauma to the mucosal lining of the airway. Swabs were placed in the barrel of a sterile 12-cc syringe and held on ice until centrifugation. Nasal secretions were collected into the bottom of a polypropylene, 12-cc syringe casing by centrifugation at 4000 rpm for 20 min at 4° C. The samples were transferred into 1.8-mL cryogenic storage vials and placed in a - 20° C freezer until assayed.

Figure 3-2. Placement of rayon-tipped proctoscopic swabs in horses to collect nasal secretions for IgA analysis. Swabs were inserted approximately 15-cm-deep into the ventral meatus where they remained for 5 min. Nasal secretions were recovered from the swabs by centrifugation described in materials and methods.

Absorption of Antibody by Rayon Swabs

To determine the amount of antibody and fluid volume retained by Rayon swabs, a pre-measured volume containing a known antibody titer was adsorbed onto duplicate swabs and the eluted fluid was assayed for antibody titer. A test suspension containing a 1:10 dilution of equine-specific IgGa monoclonal antibody was assayed prior to and after centrifugation through the swab at 4000 rpm for 20 min at 4° C in a manner identical to serum samples described in the previous section on ELISA development. The fluid weight and volume were measured before being absorbed onto the swab and after centrifugation. The amount of antibody retained in rayon swabs was determined by 1) measuring the OD reading from an ELISA using primary antibody supernatant at a 1:10 dilution and 2) obtaining the absorbance of the suspension at 280 nm. One method of quantifying protein is to measure the absorbance of UV irradiation by proteins at 280 nm. An approximation of the concentration of protein in a solution is determined by the relationship, 1 absorbance unit is equal to 1 mg/mL (Peterson, 1983).

Results

Validation of Infection of Horses with Equine Influenza Virus

The techniques and the virus strain used to infect horses resulted in clinical disease (Table 3-2) and seroconversion (Figure 3-3). The mean clinical score for the high-dose group was 21.5 and 18 for the low-dose group. In this experiment, six of six horses infected with a "high dose" (10^8 EID_{50}/animal) and six of six horses infected with a "low dose" (10^5 EID_{50}/animal) of EIV-Kentucky-95 shed virus for an average of 7 and 6 days respectively. The maximum titer of virus shedding was $10^{6.5}$ EID_{50}/mL and 10^5 EID_{50}/mL for high and low challenge-dose, respectively. The maximum titers of viral shedding occurred two to three days later in the low-dose than the high-dose. The non-infected controls did not shed virus.

Table 3-1. Mean clinical scores of horses infected with a "high" (EID_{50} 10^8) and "low" (EID_{50} 10^5) dose of equine influenza virus.

Group ID	Animal #	Animal Total	Group Mean
High Dose	44	24	21.5 ± 17.4
	45	24	
	46	29	
	47	8	
	48	57	
	50	11	
Low Dose	54	23	18 ± 4.1
	55	16	
	56	16	
	57	22	
	58	19	
	59	12	
Negative Controls	46	0	< 1
	49	0	
	52	2	
	51	0	

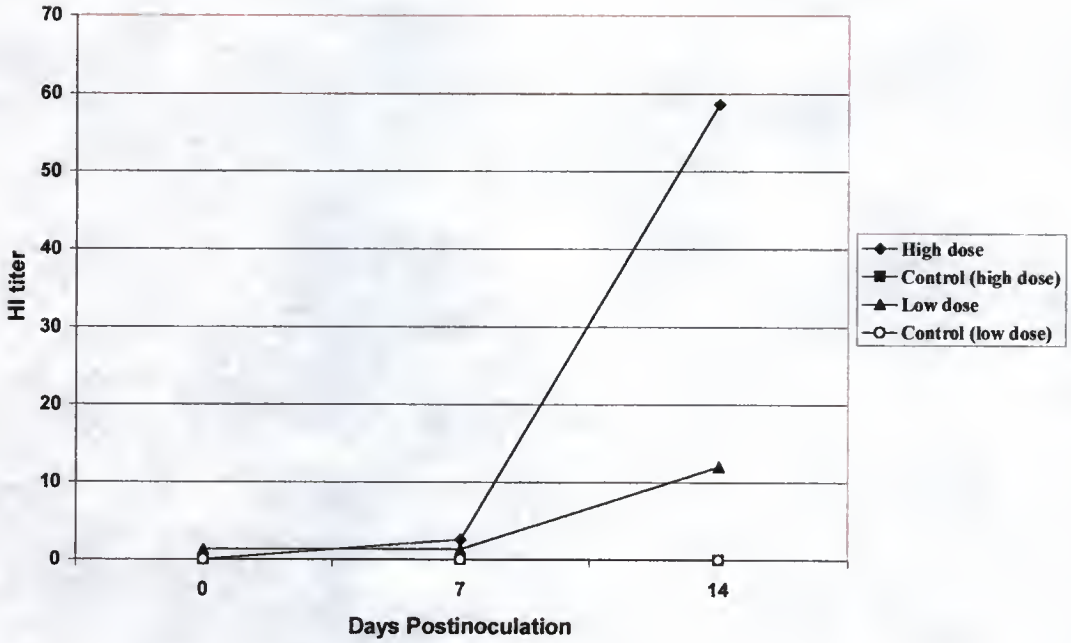


Figure 3-3. Serconversion of horses infected with a "high" (EID_{50} 10^8) and "low" (EID_{50} 10^5) dose of equine influenza virus. (High dose n = 6; Low dose n = 6; Controls n = 2)

Sampling Method Used to Collect Nasal Secretions

Horses tolerated the placement of swabs into each nostril for the 5-min sampling period without the use of a sedative. The swab-tips were small enough so as not to occlude breathing. A clear non-viscous nasal discharge was sometimes noticed dripping from the nostril when the swabs were in place. No hemorrhaging was noticed in association with the placement and removal of the swabs. From a total of 138 sampling events, the average volume of secretions was 0.27 mL and 0.54 mL per nostril and horse respectively (Figure 3-4).

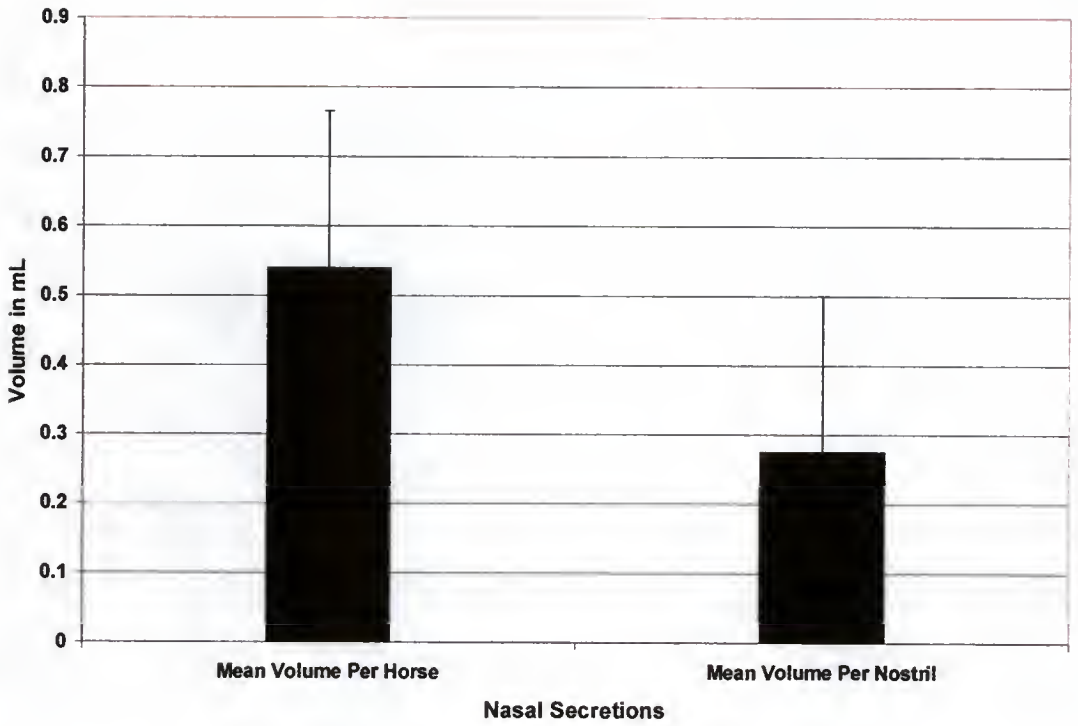


Figure 3-4. Mean volume of nasal secretions from horses that were collected by inserting rayon-tipped swabs into the nostrils for 5 minutes. (Sample n = 138) Error bars represent ± SE of the mean.

Determination of Antibody Resorption by Rayon-Tipped Swabs

Approximately 10% of the fluid (corresponding to volume) was retained by the swabs in association with the techniques used to process nasal samples (Table 3-2). An ELISA did not detect a loss in antibody concentration after absorption in swabs. Furthermore, no loss of protein was detected by measuring the absorbance of the antibody-containing fluid at 280 nm (Table 3-2).

Table 3-2. Fluid and Antibody Retention of Rayon Swabs

Swab #1		Swab # 2	
Fluid wt. Before	1.88 gm	Fluid wt. Before	1.94 gm
Fluid wt. After	1.67 gm	Fluid wt. After	1.7 gm
Fluid wt. Retained	0.16 gm	Fluid wt. Retained	0.23 gm
% Retention	8.5%	% Retention	11.8%

Anti-equine IgG	Mean ELISA OD	Absorbance at 280 nm
Before Absorption	1.041	1.315
After Fluid Recovery	1.155	1.556

Discussion

Studies performed at the Animal Health Trust in the U.K. typically include administering nebulized virus into a room, (Mumford, Hannant, & Jessett, 1990). In the present study, individual horses were infected with EIV using a nebulizer attached to an AeroMask™. This method has potential advantages over other methods by reducing the risk of environmental contamination and spread of virus. Horses tolerated the placement of the mask which allowed the direct administration of aerosolised virus particles into the upper air ways. The infectious dose and method of administration resulted in clinical disease similar to that reported by others

(Mumford, Hannant, & Jessett, 1990). It was interesting to note that while both high and low dose groups shed maximum titers that were similar, the peak occurred two to three days later in the low-dose group. This may have been partially due to the additional time required for viral replication in the low-dose group to catch up to that seen in horses receiving the high-dose. Based on these data, a challenge dose of 10^8 EID_{50}/animal of EIV was used to challenge horses in the animal study (Chapter 4).

Local antibody formation is believed to be critical for protection against EIV. In recent studies to determine the level of IgA formation in horses following vaccination and infection, tampons were placed in the nostrils of ponies. However, the tampon method requires the sedation of horses. An alternative to the tampon method was employed by placing rayon-tipped swabs 15-cm-deep into the ventral meatus for five min. While this procedure clearly caused discomfort, no horses required sedation. During the 5-min sampling-period, an average volume of 0.54 mL of nasal secretions was collected from each horse. The ELISA protocol required only 10 µl of sample per run. Therefore, this method proved to be adequate in obtaining a sufficient volume of nasal secretions for the study. Care was taken to avoid trauma to the mucosal surface and no hemorrhaging was associated with this procedure. This method eliminated procedures to account for an unknown dilution factor associated with lavage techniques. The alternative use of wicks or swabs over nasal washes was reported to have higher IgA yields in humans (Thompson, Pham et al., 1996). In the current study, proctoscopic swabs did not retain a significant volume of fluid after centrifugation. Furthermore, the amount of antibody lost due to adherence was below the ELISA detection limits. The method to determine antibody loss included anti-equine IgGa diluted in PBS. This did not account for potential variation associated with the mucous component of nasal secretions, however. Based on the results of this study, the use of rayon-tipped proctoscopic swabs to absorb mucosal fluid from horses was concluded to have advantages over nasal-lavage and as effective as the tampon method without the sedation of horses.

CHAPTER 4

ANIMAL STUDIES

Introduction

Equine influenza virus is known to cause upper respiratory disease in susceptible horses despite their vaccination history. This is, in part, due to the inability of conventional inactivated vaccines to induce all the components of the immune response. Further, due to the antigenic changes that occur in influenza viruses and the mobility of the equine community, horses that receive routine immunizations are nevertheless likely to encounter a variant field strain to that included in the vaccine. Collectively these events are potentially responsible for maintaining the endemic nature of this disease. The importance of serum and nasal antibody formation in horses to prevent infection or reduce the severity of clinical disease has been well established (Mumford & Wood, 1992) (Lunn, Soboll, Schram, Quass, McGregor, Drape, Macklin, McCabe, Swain, & Olsen, 1999). In addition, others have described the importance of the cell-mediated arm of the immune response during infection with EIV(Hannant & Mumford, 1989). Others have reported the antigen-specific proliferation and cytotoxic T-lymphocyte response induced from either vaccination or infection with EIV (Hannant & Mumford, 1989;Hannant, 1994). Several reports have characterized serum or local antibody increases in horses after vaccination with various adjuvanted inactivated viruses (Wood, Mumford, Folkers, Scott, & Schild, 1983;Mumford, Wood, Folkers, & Schild, 1988;Mumford, Jessett, Dunleavy, Wood, Hannant, Sundquist, & Cook, 1994a;Mumford, Wilson, Hannant, & Jessett, 1994;Mumford, Jessett, Rollinson, Hannant, & Draper, 1994b), modified-live virus (Holmes, Lamb et al., 1991;Lunn, Hussey, Sebing, Rushlow, Radecki, Whitaker-Dowling, Youngner, Chambers, Holland, Jr., & Horohov, 2001),

and DNA vaccines (Lunn, Soboll, Schram, Quass, McGregor, Drape, Macklin, McCabe, Swain, & Olsen, 1999;Nelson, Schram, McGregor, Sheoran, Olsen, & Lunn, 1998). However, no studies describing cytokine mRNA expression in horses following vaccination against or infection with EIV have been conducted.

In the present study, a comprehensive approach is described to characterize the immune response, including systemic and local antibody formation, cell mediated immunity, and the induction of cytokines following immunization with a novel recombinant DNA vaccine. In addition, a series of identical experiments using a modified-live and inactivated-virus vaccine were conducted as a comparison. Each vaccine was further assessed for its ability to prevent infection or severe clinical disease when compared to that seen by natural infection.

Materials and Methods

Horses

Sixteen Belgian and Percheron draught horses of both sex and approximately 12 months-old were purchased from a farm in Manitoba, Canada. Prior to their purchase, horses were bled and sera were screened for antibodies to either EIV or EHV-1 and 4. Horses that were identified as being sero-negative to EIV (by HI and SRH) and EHV (by complement fixation assay) were purchased and transported to the UF equine research center in Ocala, Florida. To avoid the potential of exposure to viral pathogens typically found in stock-yards, the draught horses were watered and fed in the trailer. An HI and an ELISA were performed on each animal again prior to enrolling them in the study. All studies were approved by the UF Institutional Animal Care and Use Committee (IACUC).

Vaccines

Three vaccines were used in this study. The recombinant DNA vectored vaccine (Hoescht Roussel Vet, Marburg, Germany) was an experimental product being tested in our laboratory for efficacy against infection with EIV. The EHV-4 (Dutta strain) "back-bone" vector (Construct S-4EHV-045), lot # UF-001 consisted of mutations in a large region of 3 genes: Thymidine kinase = 634 bp, unique short region 2 (US2) = 705 bp, and the glycoprotein E (gE) = 1696 bp (Figure 4-1). Hemagglutinin and NA genes were then inserted with the appropriate promoter into the portion of the gE gene deletion. Western blot analysis conducted in a collaborating laboratory (David Bloom, University of Florida) demonstrated HA and NA protein expression in Vero cell cultures (data not shown). The virus suspension of lysed Vero cells was determined to contain an infection titer of $10^{6.2}$ PFU/mL. Two mL of the recombinant vaccine was administered either by intranasal (IN) or intramuscular (IM) route to two different groups of horses (n=3). Intramuscular inoculation consisted of administering 2 mL of vaccine virus at the level of the fifth cervical vertebra, dorsal to the upper edge of the brachiocephalic muscle and ventral to the fundicular part of the nuchal ligament. Intranasal inoculations consisted of instilling 2 mL of the vaccine suspension using a size # 8-french polypropylene catheter (Sherwood Medical, St. Louis, MO) inserted approximately 15 cm deep into the left nostril. The horses were individually housed in an isolation stall equipped with negative airflow and HEPA filtration in a BSL-2 facility.

Fluvac® Plus (Fort Dodge Laboratories, Inc., Fort Dodge IA) is an inactivated-virus vaccine prepared with types A_1 (Prague-56) and A_2 (Kentucky-92) killed virus in combination with MetaStim® as an adjuvant. This vaccine is reported by the manufacturer to stimulate have significant neutralizing antibody increases against Alaska-91, Kentucky-91, Kentucky-93, Kentucky-94, Saskatoon-90, Kentucky-92, Kentucky-95 (type A_2 viruses), New-Market-93, New-Market2-93, Sussex-89, Arundel-91, and Prague-56 (type A_1 viruses). One mL of vaccine

was delivered by intramuscular route in the same location describe for the recombinant vaccine. Horses receiving this vaccine were held in a pasture at the UF Equine Research Farm in Ocala, Florida until challenge-infection.

Flu Avert™ (Heska, Fort Collins, CO) is a modified-live intranasal vaccine containing a cold-adapted strain of Kentucky-91 (type A_2 virus). This vaccine is reported by the manufacturer to protect against infection with Kentucky-91, Kentucky-98 (American A_2) strains and Saskatoon-90 (Eurasian A_2 strain). The vaccine is supplied as a desiccated virus that requires re-constitution with sterile water. In compliance with the manufacturer's recommendations, the vaccine was reconstituted less than ten min prior to a 15-cm-deep intranasal inoculation. Animals receiving this vaccine were held in a pasture at the UF, Equine Research Farm in Ocala, Florida until challenge-infection. This vaccine was determined in our laboratory to have an HA titer of 1:64.

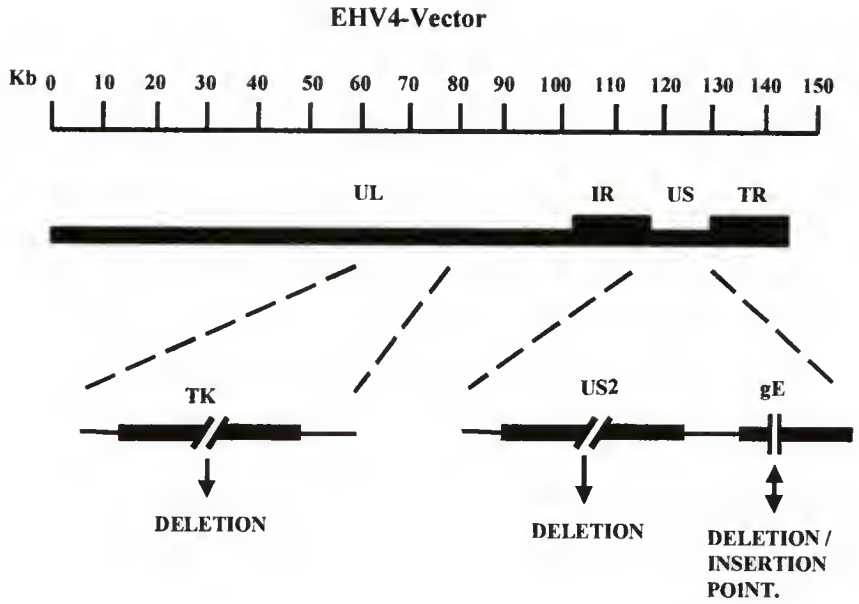


Figure 4-1. Schematic representation of the recombinant DNA vaccine EHV-4 "backbone" vector showing gene deletion and insertion points. The genes for the EIV proteins, HA and NA, were inserted at the gE deletion site.

Viruses

The EIV strain A/equine/2/Kentucky/95 was obtained from Dr. Tom Chambers (University of Kentucky) at the second egg-passage. The virus was passaged in eggs a further time and frozen at –70° C until used. The virus was tested for influenza by hemagglutination and titrated in ten-day-old embryonated chick eggs. The A/equine/2/Kentucky/91 strain contained in the modified-live vaccine was passaged in ten-day old embryonated chick eggs prior to its use in proliferation assays.

Sample Collection and Storage

Serum

Whole blood was collected by jugular venipuncture into 7-mL capacity red-top, serum-separator tubes (Fisher Scientific, Atlanta, GA) using a 20-g needle. Blood was allowed to separate for 1 hr at room temperature prior to centrifugation at 2500 rpm for ten min at 4° C. Sera were stored in four, 1.8-mL cryogenic storage tubes (Nunc, Nenmark) at -20° C in two separate freezers until assayed.

Peripheral blood mononuclear cells

Whole blood was collected by jugular venipuncture into heparinized green-top tubes. Peripheral blood mononuclear cells (PBMC) were isolated as previously described (Allen, Yeargan, Costa, & Cross, 1995) with a FICOLL-Histopaque 1.077 g/mL density gradient (Sigma Chemical Co., St. Louis, MO). The cells were washed three times in phosphate buffered saline (PBS) (Gibco BRL, Grand Island, NY) and pelleted by centrifugation at 250 x g for ten min at room temperature. One mL of red blood cell (RBC) lysing buffer (8.3 g/L ammonium chloride in 0.01 M Tris-HCL buffer) (Sigma Chemical Co.) was added to the cell pellet if RBC were present. The PBMC were washed an additional two times if RBC lysis buffer was used. For long-term storage, PBMC were suspended in a freezing medium consisting of RPMI-1640 with 10-mM

Hepes buffer, 2-mM L-glutamine, .075% w/v sodium bicarbonate, 1 mM sodium pyruvate, 100 U/mL penicillin-G, 100 µg/mL streptomycin, 10% BFS, and 50% bovine fetal serum (BFS) (Gibco BRL, Grand Island, NY) at a density of approximately 1 x 10^7 cells/mL. The PBMC were placed in a Nalgene™ Cryo 1° C freezing canister (5100-001) containing 70% isopropyl alcohol. The container was placed in a – 80° C freezer over-night to achieve a controlled rate of freezing. The tubes containing frozen cells were subsequently transferred to liquid nitrogen (cells were stored in the liquid phase).

Nasal secretions for IgA antibody determination

Horses were sampled by methods described in chapter three under standardization and development of animal infection and sampling methods.

Experimental and challenge infection

Horses assigned to the EIV-infection group received an initial infection at an infectious dose of $10^{6.5}$ EID_{50} on post infection day (PID) zero. Horses were housed in individual stalls within Progress Center's large-animal (biosafety level 2 facility) research barn during the infection procedure and up to ten days afterward. Individual horses within the vaccine and EIV-infection groups were challenged with an infectious dose of 10^8 EID_{50}/animal by methods described in chapter 3.

Virus isolation

A 16-in rayon-tipped proctoscopic swab was inserted approximately 15-cm-deep into the ventral meatus of each nostril where it remained for 15 sec. The swabs were placed into polystyrene tubes containing viral transport medium (VTM). The VTM was composed of Dulbecco's Modified Eagle Medium with 25 mM Hepes, 5% BFS, penicillin-G sodium (100

units/mL), streptomycin sulfate (100 μg/mL), amphotericin B (0.25 μg/mL), and gentamicin sulfate (0.1 mg/mL) (Gibco, Grand Island, NY). The swabs were kept on ice until they were processed. The swabs were removed from the tubes and placed in a sterile 12-cc syringe barrel, which in turn was placed in a sterile 12-cc syringe casing and centrifuged at 1000 x g at 4° C for 5 min. The swab extracts were placed in duplicate 1.8-mL cryogenic storage vials and placed in a – 80° C freezer.

Clinical signs

A clinical score was assessed to each animal during the sampling period based on various physical exam parameters listed in Table 4-1. Daily assessment was made by persons not blinded to the treatment group.

Table 4-1. Scoring system associated with clinical signs observed in horses following infection with equine influenza virus

Parameter	Observation	Assessed Value
Rectal Temperature in Degrees F	**98.5-101.9**	**0**
	102.0-104.0	**1**
	103.1-105.0	**2**
Nasal Discharge	**Yes**	**1**
	No	**0**
Anorexia	**Yes**	**2**
	No	**0**
Coughing	**Yes**	**1**
	No	**0**
Lethargy	**Yes**	**1**
	No	**0**

Daily observations to assess clinical illness were made within one hour of an established time throughout the study. The duration of data collection included seven days prior to the start of the

study. No treatment intervention related to vaccination or challenge-infection with EIV was required during the study.

Virus Isolation and Titration

To determine the presence and amount of viral shedding from the upper respiratory tract, nasal samples were collected by methods described in chapter 3. One of two aliquoted samples was rapidly thawed in a 37° C water bath. Samples were held on ice during dilution steps. The amount of virus in the test samples was determined as previously described (Hsiung, 1994). Ten-fold serial dilutions of the nasal samples were made in virus transport medium (VTM). Virus transport medium consisted of DMEM with 25 mM Hepes, 5% BFS, penicillin-G (200 U/mL), streptomycin sulfate (200 μg/mL), amphotericin B (0.5 μg/mL), and gentamicin (0.2 mg/mL). A 0.1 mL volume of neat and serially diluted samples was injected, in triplicate, into the allantoic cavity fluid of 10 dy-old embryonated chick eggs using a 22-ga needle and tuberculin syringe. Three eggs were injected with 0.1 mL of a 1:5 diluted allantoic fluid containing EIV Kentucky-95 at a HA titer of 1:128 in VTM as positive controls. Three eggs were injected with VTM alone as negative controls. The eggs were incubated for three days at 34° C. The presence of virus in each egg was determined by the addition of 50 μl of allantoic fluid to 50 μl of a 1.0 %-suspension of chick red blood cell (CRBC) in a 96-well, Nunc V-bottom microtiter plate (Fisher Scientific, Suwanee, GA). The mixture was allowed to incubate at room temperature for 30 min. Hemagglutination of CRBC by virus was assessed by tilting the plates at a 70° angle. The absence of "streaming" in the test samples and positive controls indicated the presence of influenza virus. Positive and negative controls included the addition of 50 μl of allantoic fluid containing Kentucky-95 or PBS alone to test wells respectively. The egg infectious dose 50 (EID_{50}) was calculated by the Reed and Muench method (Reed & Muench, 1938).

Serology

Hemagglutination inhibition, SRH, and ELISA testing methods were conducted as previously described in chapter 2.

Nasal Antibody Determination

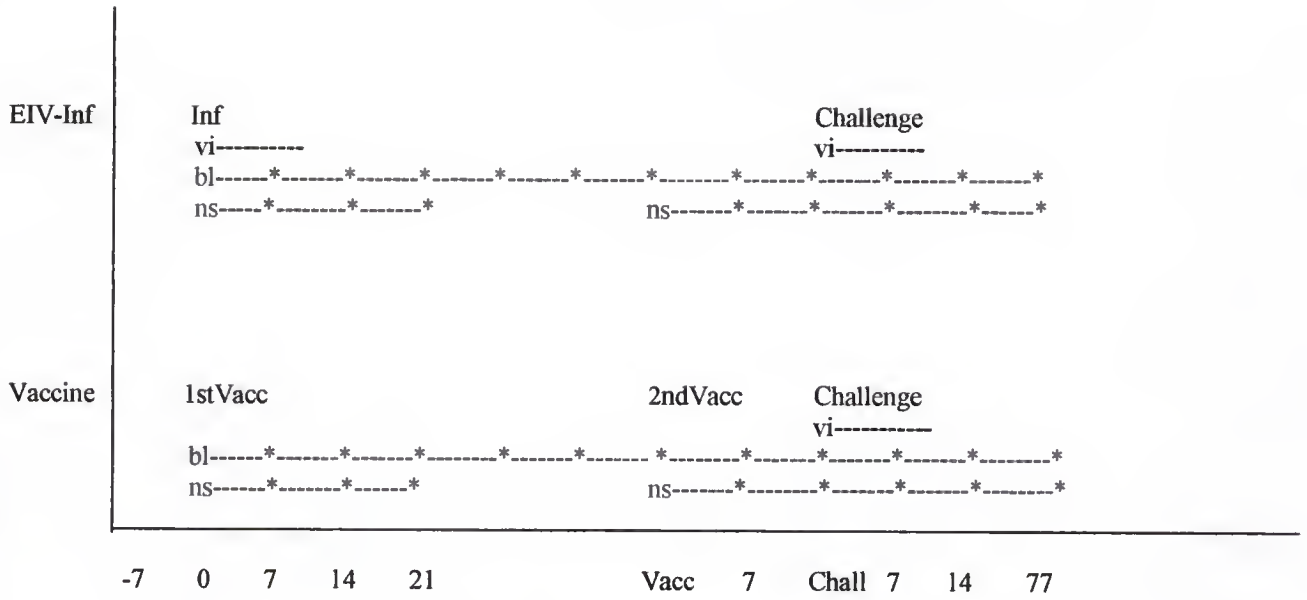
Nasal antibody was determined by techniques described in chapter 2.

Cell-Mediated Immunity

Antigen-specific proliferation assays and induction of cytokine mRNA by Realtime PCR techniques were completed to determine CMI by methods described in chapter 2.

Study Timeline

Vaccinates were placed in groups of three animals each and were inoculated on day zero and day 42 and were challenged on day 56. Horses within the EIV-infection group were infected on day zero and challenged on day 63. Nasal secretions, and virus isolation samples were collected as using methods described in chapter 3.



Definition of groups and sample collection

EIV	Horses infected with live equine influenza virus
Vaccine	Horses immunized with vaccines
vi	Virus isolation
bl	Blood collection
ns	Nasal secretions

Figure 4-2. Timeline for vaccination, infection, and sampling of horses. EIV-infected group was infected on day zero and challenged 8 weeks later. Vaccinates were immunized on day zero and 42 and challenged on day 56. Blood, nasal, and virus isolation samples were collected on days designated by *. (EIV n = 3; Vaccinates n = 3 per group; un-vaccinated control n = 1)

Statistical Analysis of the Immune Response in Vaccinated and Infected Horses

Within individual groups, comparison of data over time was related to changes over that seen on day zero (baseline values). Daily comparisons were made within individual groups, and between groups on given sample days, using a two way repeated measures analysis of variance (one factor repetition) method. Pairwise multiple comparisons were analyzed by the Student-Newman-Keuls method using Sigma-Stat software. For all comparisons, significance was considered where alpha was less than 0.05. Two by two tables were constructed to determine the

relationship between serum antibody and clinical disease and nasal antibody and virus shedding. A Fishers exact test was used to determine the statistical significance in the proportion of animals with an association between antibody formation and protection against clinical disease and infection. A Pearson correlation coefficient (r) with a 95% confidence interval was calculated to determine the relationship between nasal IgA formation and a reduction in viral shedding on the peak day of virus shedding.

Results

Clinical Scores

Clinical scores for each group are summarized in Table 4-3. There was no significant difference in the mean clinical scores between vaccine groups. Horses within the recombinant DNA vaccine group were not protected against infection. This was reflected in their clinical scores of seven and 11.3 for the IM and IN vaccinates, respectively. Horses receiving the MLV vaccine showed a reduction in clinical signs of clinical disease compared to that of the unvaccinated control with a group mean score of 5.3. The CS values in the MLV vaccine group were mostly elevated due to a single horse (#148). Horse number 148 had bilateral nasal discharge, coughed frequently, and had an elevated temperature after challenge-infection resulting in a total CS of 15. Horses receiving the inactivated vaccine had a lower mean CS than the MLV vaccinates. Horses in the EIV-infection group had a mean clinical score ranging from two to seven after the initial exposure on day zero. However, these horses showed no clinical disease after challenge-infection with a group mean of zero. The un-vaccinated control had a clinical score of 13.

Table 4.3 Clinical scores for vaccinated and infected horses following challenge with equine influenza virus. Horse # 147 was not challenged. Clinical scores are based on criteria presented in table 4-1

Group ID	Animal #	Animal Total	Group Mean
Recomb IM	131	5	7 SD ± 1.7
	134	8	
	135	8	
Recomb IN	133	13	11.3 SD ± 3.8
	136	14	
	137	7	
EIV-Infection	114	0	0.0 *
	119	0	
	120	0	
MLV	146	1	5.3 SD ± 8.4
	148	15	
	151	0	
Inactivated	147	NC	1 SD ± 0
	149	1	
	150	1	
Positive Control	132	13	13.0

Virus Shedding in Vaccinated and Infected Horses

The average number of days and the amount of virus shed varied between experimental groups. While horses in the EIV-infection group shed virus in the range of $10^{1.7}$ to $10^{4.5}$ EID_{50}/mL on days two and three after their initial exposure, no horses shed virus upon challenge-infection, eight weeks later (Figure 4-3). Horses in the EIV-infection group were completely protected from infection and thus, significantly different from the recombinant ($P<0.001$), MLV ($P=0.003$) vaccinates, and negative control ($P=0.008$).

Eleven of 13 vaccinated horses challenged with A/equine/2/Kentucky/95 shed virus. There was a significant difference ($P<0.05$) in the level of virus excretion between the recombinant and commercial vaccine groups on day three, after challenge. Consistent with other infection challenge studies conducted in our laboratory, horses in this study, had a peak in their virus shedding on day three, post infection. Horses receiving the IM and IN recombinant vaccine shed virus from days one through six post-infection at titers ranging from $10^{1.3}$ to $10^{3.5}$ and $10^{1.5}$ to $10^{5.8}$ EID_{50}/mL respectively. Total virus shedding for the post-infection sampling period is represented in figure 4-4. Nasal isolates from the IM and IN recombinant vaccine group were negative for EHV-4 virus for the 1- days following infection (data not shown). Horses that were vaccinated with the MLV or inactivated virus vaccines shed virus from days one through five post-infection at titers ranging from $10^{.75}$ to $10^{4.75}$ and $10^{.75}$ to $10^{2.24}$ EID_{50}/mL respectively. The total amount of virus shedding in the MLV and inactivated-virus vaccinates for the post-infection sampling period is represented in figure 4-5. Horse number 148 within the MLV vaccine group presented with severe clinical signs and shed virus for a greater number of days than other horses. However, using a two way ANOVA, there was no significant difference in virus titers between animals in this group. Horse number 150, within the inactivated-vaccine group, did not shed virus after challenge-infection.

Figure 4-6 summarizes the results of virus isolation in the each group. While nasal secretions were collected for ten days post-infection, virus was not shed past six days in any of the groups. There was no significant difference in the maximum number of days of virus excretion for any of the vaccine groups.

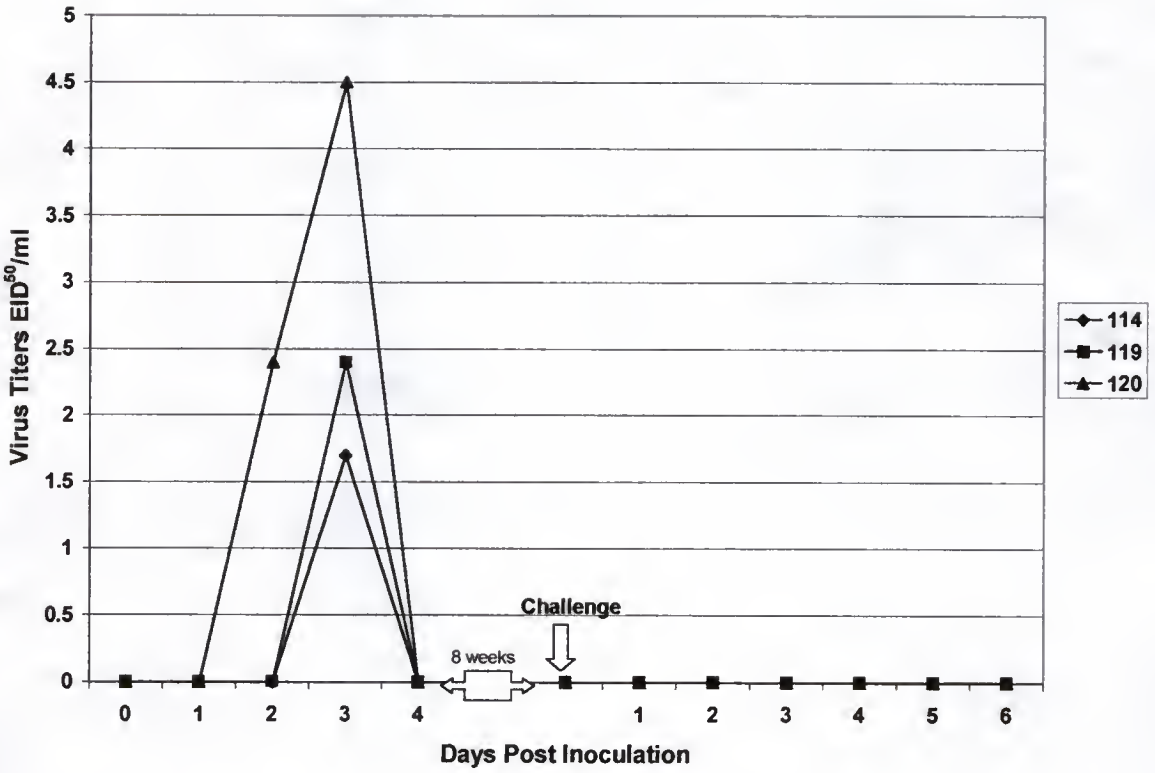


Figure 4-3. Viral shedding in horses in the EIV-infection group (EIV/INF) initially infected with equine influenza virus at an EID_{50} of $10^{6.5}$ followed by a subsequent challenge, 8 weeks later.

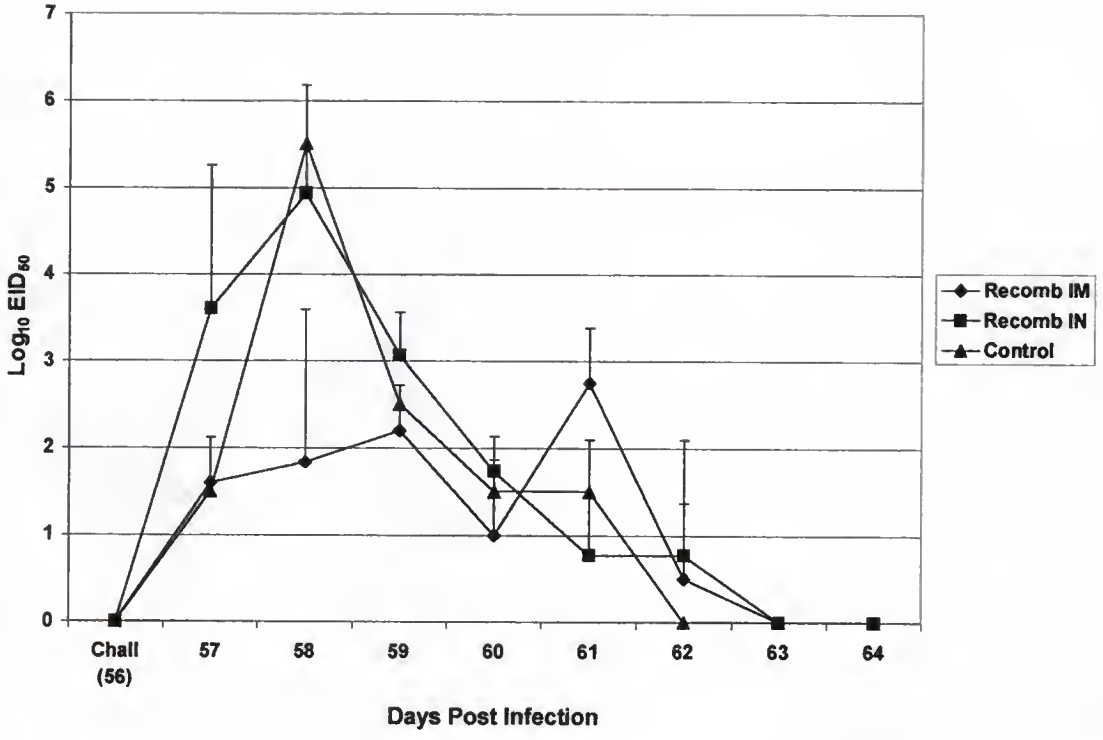


Figure 4-4. Viral shedding in the IM and IN recombinant (Recomb) vaccinates after challenge with A/equine/2/Kentucky/95 EIV at an EID_{50} of $10^{8.0}$. Recombinant IM and IN, n = 3. Control is un-vaccinated horse, n = 1. Error bars represent the standard deviation from the mean.

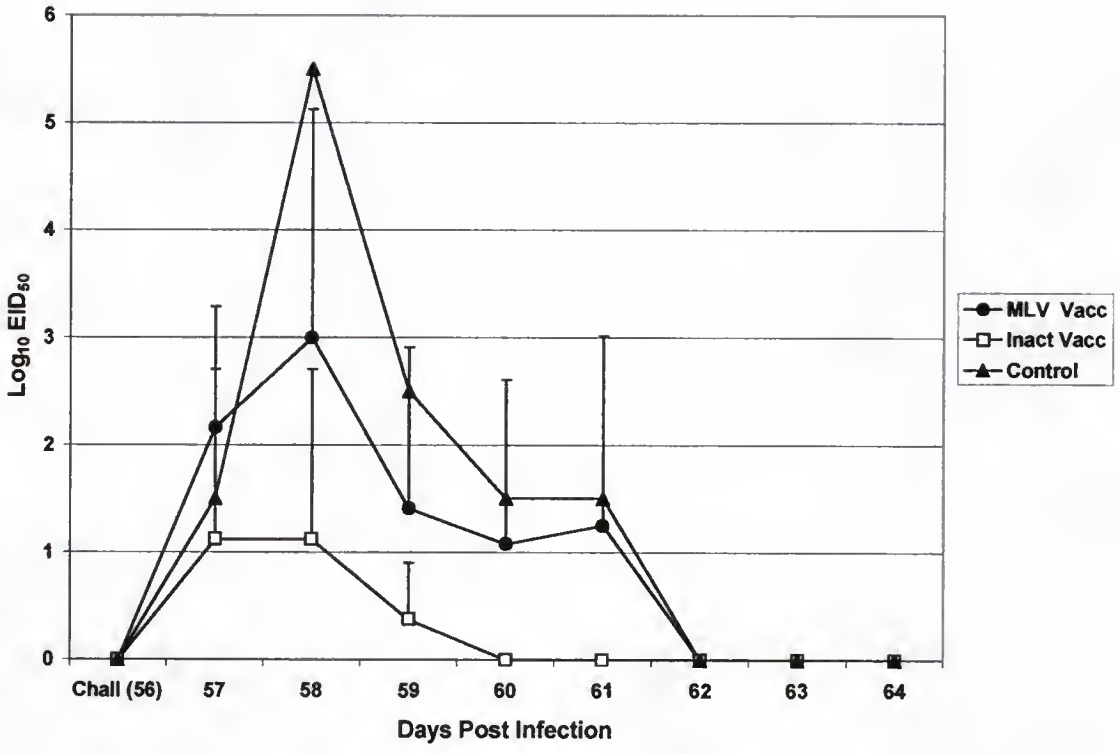


Figure 4-5. Viral shedding in the modified live (MLV) and inactivated-virus (INAX) vaccinates after challenge with A/equine/2/Kentucky/95 EIV at an EID_{50} of $10^{8.0}$. MLV and inactivated, n = 3. Control is un-vaccinated horse, n = 1. Error bars represent the standard deviation from the mean.

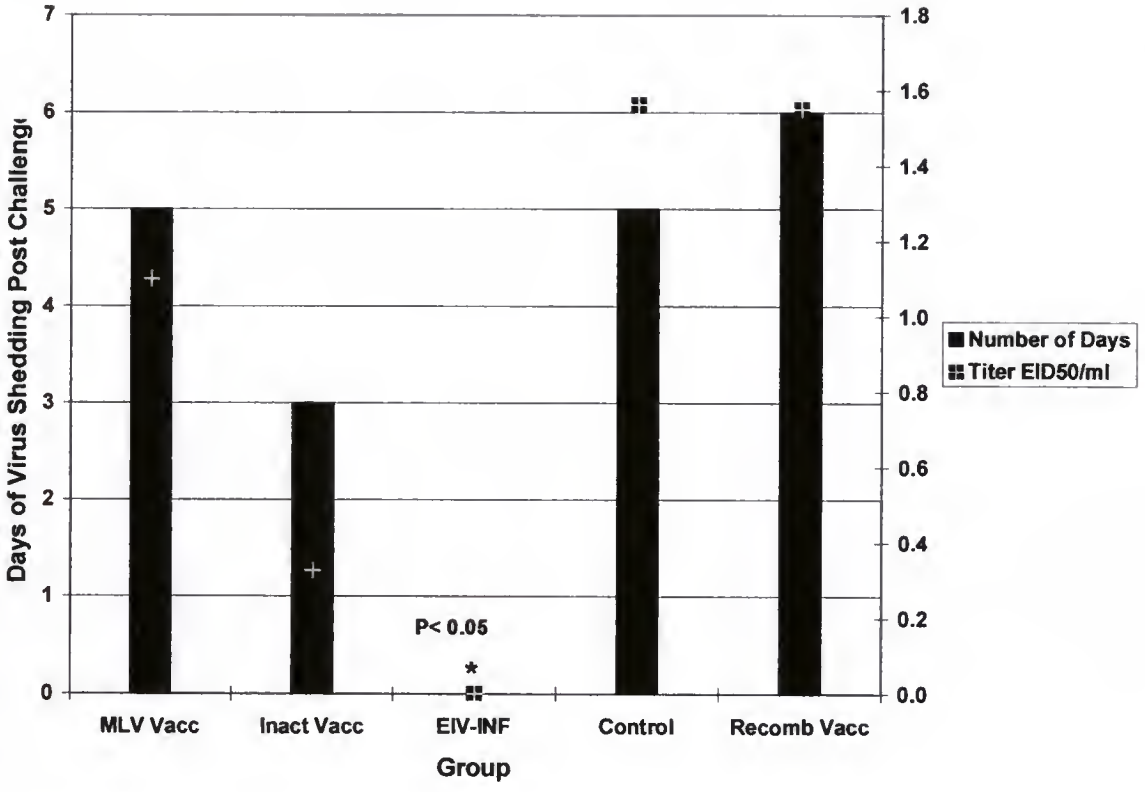


Figure 4-6. Summary for both the maximum number of days and titers of virus shedding for vaccinated and infected horses.
* Indicates a significant difference $p< 0.05$ after group-wise comparison by two way analysis of variance. (Vaccine and EIV-INF groups n = 3; Control n = 1)

HI

Hemagglutinin inhibition antibody titers to homologous challenge were a) greater at an earlier point after experimental infection and b) persisted at higher levels for a longer duration in the EIV-infection group when compared to the response induced by vaccines or the negative control (Figure 4-7).

The IN and IM recombinant vaccine failed to induce HI antibodies after the initial or booster inoculation. A rise in the HI titer was not detected in these groups until seven days following challenge-infection; neither of which was significantly higher than the negative control. Two of three horses immunized with the inactivated-virus vaccine showed an increase in HI titers at day seven which peaked by day 28. By day 56 (challenge infection), one of three horses in this group appeared to have HI antibody levels that, while not significantly higher than any other group, are considered to be protective against severe clinical disease. Horses receiving the cold-adapted vaccine did not show an appreciable rise in HI titers until 14 days after challenge infection. Horses infected with virus on day zero seroconverted by day 14 whereas those receiving the inactivated virus vaccine did not reach the equivalent HI titer until day 28. Furthermore, only those horses within the EIV-infection group maintained HI titers considered adequate to protect against severe clinical disease by the time they were challenged.

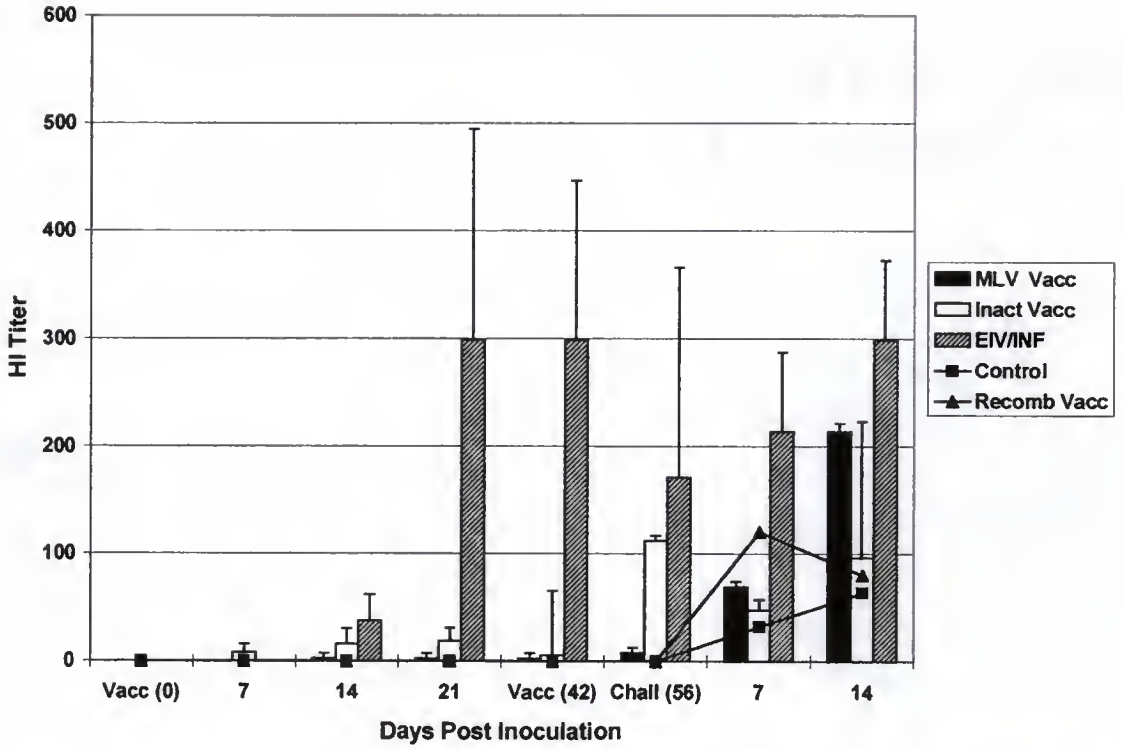


Figure 4-7. Hemagglutination inhibition antibody levels in vaccinated and infected horses. MLV, inactivated, and recombinant vaccine groups were inoculated on day zero and again on day 42. The EIV/INF group was initially infected with equine influenza virus on day zero. All groups including, the un-vaccinated control, were challenged (chall) with the Kentucky-95 strain of EIV on day 56. A four-fold increase in the HI titer indicates seroconversion. Error bars represent ± SD from the mean. (Vaccine and EIV-INF groups n = 3; Control n = 1)

SRH

A summary of the SRH antibody formation in each group is included in Figure 4-8. The recombinant vaccine failed to induce a detectable antibody response. However, horses within this group seroconverted on day seven after challenge. Single radial hemolysis antibody titers in the recombinant vaccinates on days seven and 14 after challenge were not significantly different from those in horses in the EIV-infection group following their initial exposure to live virus. Horses receiving the modified live or inactivated virus vaccines showed a similar pattern of antibody response to that seen by the HI assay method. Seven days after the first vaccination, both MLV and inactivated virus vaccines resulted in seroconversion at similar levels (equal to or exceeds 75 mm^2). Initial and booster immunizations with the inactivated virus vaccine appeared to induce a significant ($P<0.01$) increase in SRH titers 14 days after each, respectively. A further significant rise in antibody was detected on the seventh ($P= 0.04$) and 14th ($P=0.002$) day after challenge. A similar pattern of antibody increase was noted in the horses receiving the MLV vaccine. However, the MLV virus vaccinates did not show a significant rise ($P< 0.01$) until 14 days after challenge (Figure 4-8). Neither the inactivated nor the MLV virus vaccine resulted in SRH titers considered to prevent infection (140-150 mm^2 zone of hemolysis) at the time of challenge. Group by group comparison revealed a statistically significant ($P=0.008$) difference in SRH titers in the MLV vaccinates on PID 14 compared to the recombinant vaccinates. Likewise, significant differences were seen between the EIV-infection group titers and the recombinant vaccinates on day 14 ($P=0.005$) and 56 ($P=0.01$). In summary, SRH antibody formation was highest and persisted longer in horses receiving the inactivated virus vaccine when compared to those either inoculated with the recombinant or MLV vaccine.

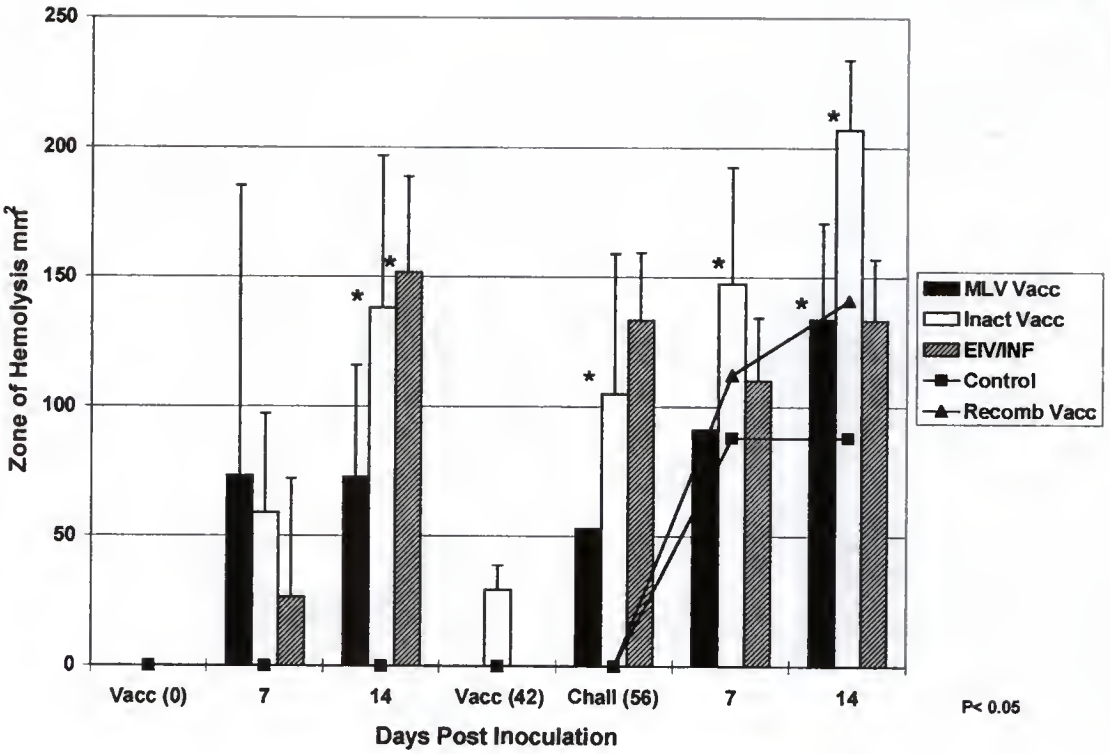


Figure 4-8. Mean single radial hemolysis antibody titers in vaccinated and infected horses. MLV, inactivated, and recombinant vaccine groups were inoculated on day zero and again on day 42. The EIV/INF group was initially infected with equine influenza virus on day zero. All groups including, the un-vaccinated control, were challenged (chall) with the Kentucky-95 strain of EIV on day 56.

* Indicates a significant difference $p<0.05$ over baseline values. Error bars represent ± SD from the mean. (Vaccine and EIV-INF groups n = 3; Control n = 1)

ELISA

A summary of the ELISA data for IgGa antibody production is shown in figure 4-9. Day zero baseline values of serum IgGa antibody among horses were not significantly different. The ELISA data had a similar pattern to that seen by the HI and SRH methods. In both the IN and IM recombinant vaccine groups, zero of six horses established an antibody response as a result of vaccination. Seroconversion was not detected in the recombinant vaccinates until seven days after challenge. One of three horses receiving the MLV vaccine and three of three receiving the inactivated-virus vaccine showed a serum IgGa antibody response as early as seven days post-inoculation. Furthermore, antibody production increased appreciably again after challenge-infection in the inactivated-virus and MLV vaccinates. Horses receiving the MLV vaccine had significant ($P = 0.05$) increases in IgGa antibody titers 14 days after challenge. Two of three horses in the EIV-infection group seroconverted on PID 7 and a significant increase over baseline values of IgGa antibody was measured on PID 14 ($P=0.006$) and 21($P=0.005$). Group-wise comparisons revealed significantly ($P<0.05$) higher titers of serum IgGa antibody in the EIV-infection group over those induced in the recombinant, inactivated-virus, and MLV vaccinates on day 7, 14, and 21 after primary vaccination. However, no increase in serum antibody was noted in the EIV-infection group after challenge.

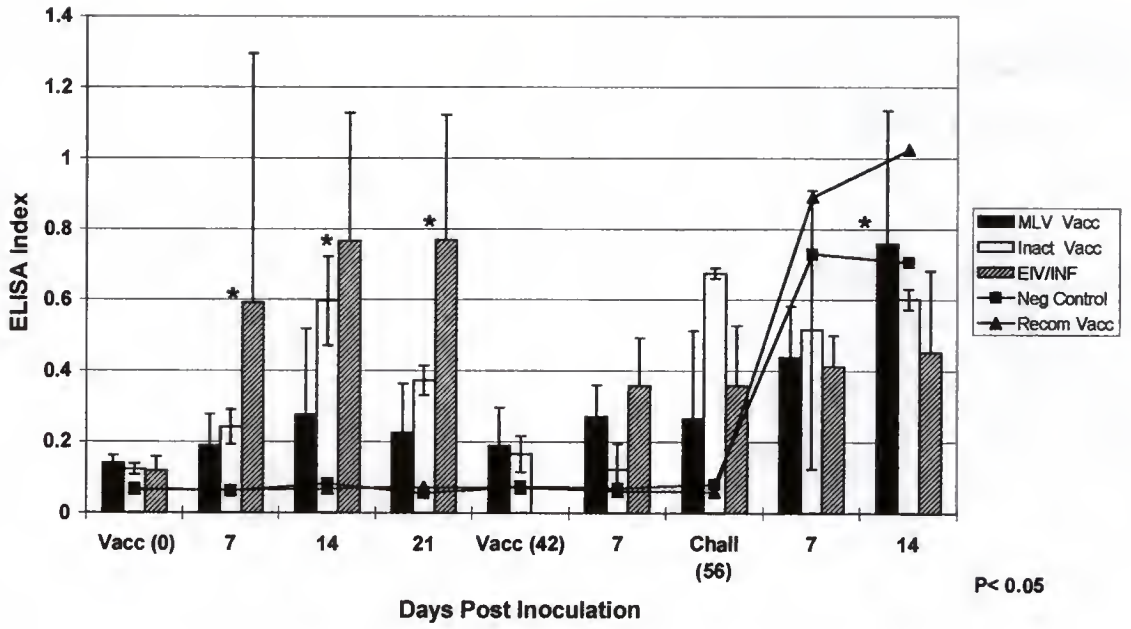


Figure 4-9. Mean serum IgGa antibody titers in vaccinated and infected horses. MLV, inactivated-virus, and recombinant vaccine groups were inoculated on day zero and again on day 42. The EIV/INF group was initially infected with equine influenza virus on day zero. All groups including, the un-vaccinated control, were challenged (chall) with the Kentucky-95 strain of EIV on day 56.

* Indicates a significant difference $p<0.05$ over baseline values. Error bars represent ± SD from the mean. (Vaccine and EIV-INF groups n = 3; Control n = 1)

Local IgA Antibody Response

The baseline values of nasal IgA antibody among horses at day zero were not significantly different. No significant ($P<0.05$) increases in local antibody production were detected by ELISA analysis in either recombinant vaccine group as a result of vaccination (Figure 4-10). However, a significant ($P=0.002$) increase in nasal IgA production was induced in the IM group after challenge-infection. Significant ($P<0.001$) increases in nasal IgA were detected in horses inoculated with the inactivated vaccine on day 14 after challenge-infection. In contrast, a significant ($P<0.001$) rise in local IgA antibody production was noted in the MLV vaccinates by the 14th day after receiving a booster inoculation and continued to increase to titers significantly ($P<0.001$) higher than baseline values through days seven and 14 after challenge. Most important, within the MLV vaccine group, nasal IgA antibody titers were not significantly different ($P=0.885$) from those seen in the EIV-infected group on the day of challenge infection. Horses in the EIV-infection group had significant ($P<0.001$) increases over baseline values by PID 14. Additionally, the level of local IgA was significantly ($P<0.05$) higher in the EIV-infection group than those produced by horses in the recombinant, inactivated or MLV vaccine groups on PID 14 and 21. However, IgA production did not increase again in the EIV-infection group after challenge-infection.

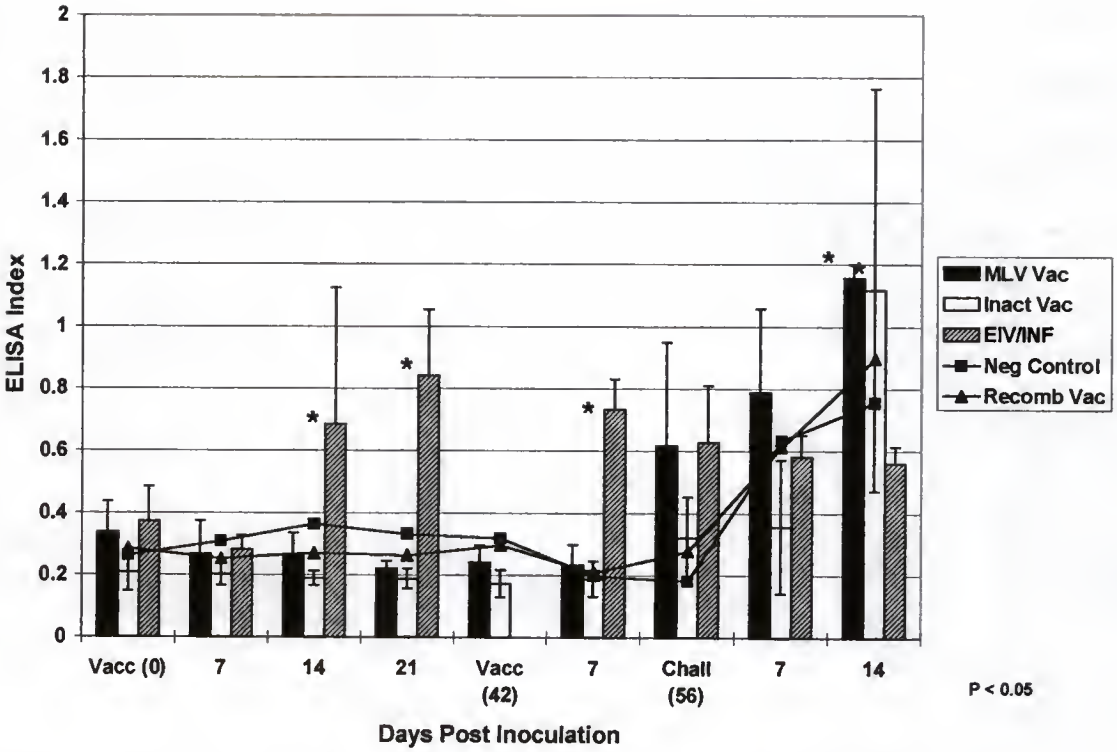


Figure 4-10. Mean nasal IgA antibody titers in vaccinated and infected horses.
MLV, inactivated-virus, and recombinant vaccine groups were inoculated on day zero and again on day 42. The EIV/INF group was initially infected with equine influenza virus on day zero. All groups including, the un-vaccinated control, were challenged (chall) with the Kentucky-95 strain of EIV on day 56.
* indicates a significant difference $p < 0.05$ over baseline values. Error bars represent ± SD from the mean. (Vaccine and EIV-INF groups n = 3; Control n = 1)

Cell-Mediated Immunity

The summary results of the antigen-specific lymphocyte blastogenesis assays for all groups of horses are summarized in (Figure 4-11). With the exception of the recombinant vaccine recipients, all other horses within this study had a stimulation index (SI) of less than one on day zero. The proliferation response was variable among horses and sample-days.

Consistent with the lack of serological evidence of antigen exposure, the stimulation indices indicated that a cell-mediated immune response was not induced by immunization with the recombinant DNA vaccine. While the mean SI for the IM and IN recombinant groups was higher at day zero, it did not increase significantly until seven days after challenge ($P<0.001$). There was a statistically significant increase in the SI over baseline values at PID 14 ($P=0.002$), 63 ($P<0.001$), and 70 ($P<0.001$) resulting from vaccination with the MLV vaccine. The SI for horses immunized with the inactivated-virus vaccine did not significantly increase over baseline values until the seventh ($P=0.018$) and again on the 14th ($P=0.002$) day after challenge. While both the inactivated and MLV vaccines contained different strains of the American-like, type-2 EIV (Kentucky-92 and Kentucky-91 respectively), both induced in a heterologous proliferative response to in-vitro stimulation with the Kentucky-95 strain of viral antigen (Figure 4-11). The proliferative potential of PBMC from both the inactivated and MLV vaccine groups was further investigated by in-vitro stimulation with the cold-adapted virus strain (Kentucky-91) contained in the MLV vaccine. There were no significant differences in the mean SI for either group of vaccinates when using the Kentucky-95 (challenge) or Kentucky-91 (cold-adapted) strain of EIV (Figure 4-12). There was a surprisingly weak response to in-vitro stimulation of PBMC from horses in the EIV-infection group after their initial exposure. Furthermore, no significant increase in the SI was detected after the challenge.

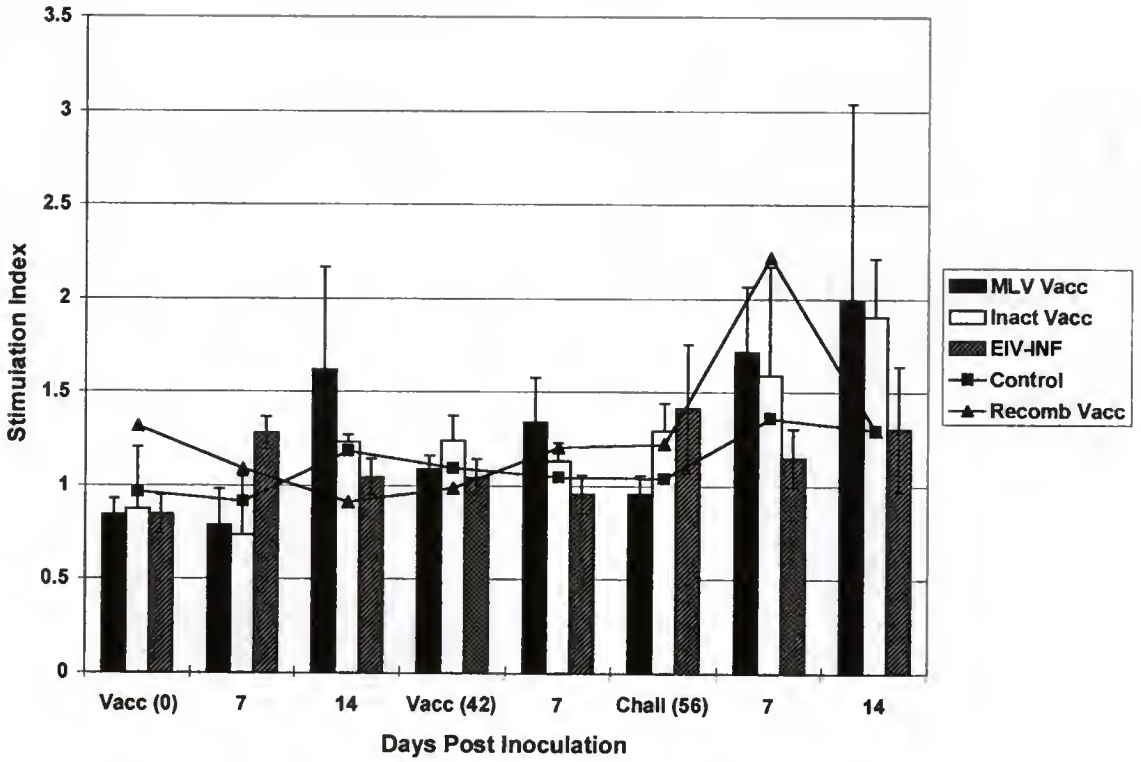


Figure 4-11. Lymphocyte proliferation response to A/equine/2/Kentucky/95 antigen. MLV, inactivated-virus, and recombinant vaccine groups were inoculated on day zero and again on day 42. The EIV/INF group was initially infected with equine influenza virus on day zero. All groups including, the un-vaccinated control, were challenged (chall) on day 56 with the Kentucky-95 strain of EIV. Error bars represent ± SD from the mean. (Vaccine and EIV-INF groups n = 3; Control n = 1)

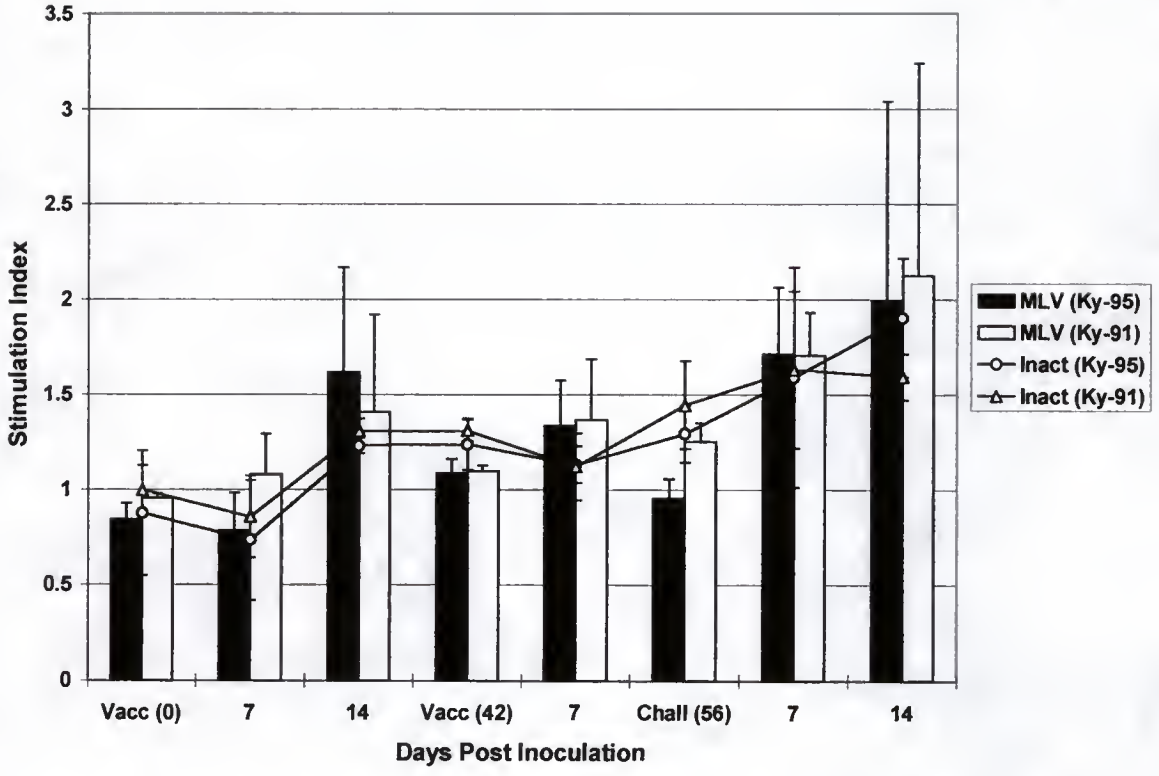


Figure 4-12. Heterotypic lymphocyte proliferation response in MLV and inactivated virus vaccinates. Peripheral blood mononuclear cells from MLV (bars) and inactivated-virus (geometric figures) vaccinates were stimulated with the A/equine/2/Kentucky/95 challenge strain of EIV (■ ○) or the A/equine/2/Kentucky/91 strain (□ △) contained in the MLV vaccine. Cells were collected from horses, 14 days after challenge with the Kentucky-95 strain of EIV. Error bars represent ± SD from the mean. (Vaccine and EIV-INF groups n = 3; Control n = 1)

Summary of Immune Responses

Tables 4-4 and 4-5 summarize the anti-influenza serum, local, and CMI response relative to clinical disease and virus shedding in all groups. Table 4-4 shows the immunological status of each horse in this study on the day of challenge (day 56). This table further summarizes the total clinical scores and virus shedding for each horse during the ten-day sampling period following challenge. Horse 147 was not challenged. Table 4-5 shows those horses that were clinically ill or shed virus and also had increases in their anti-influenza serum, local, and CMI response. Again, horse 147 was not challenged. From this table, a relationship is seen between horses that were not protected against infection and their subsequent seroconversion. Alternatively, those that did not shed virus did not seroconvert.

The data in tables 4-6 and 4-7 demonstrate the relative importance of anti-influenza serum and local antibody to both clinical disease and infection. Table 4-6 shows that anti-influenza serum antibody is associated with protection against clinical disease. In the present study, a significant proportion ($P = 0.0001$) of horses with serum antibody were protected against severe clinical disease. Nasal IgA, on the other hand, had a weak ($P = 0.11$) association with the proportion of horses that were protected against clinical disease. A "yes" response in each category was based on a test value equal to or greater than the lowest level of antibody that provided protection against clinical disease. Table 4-7 shows the importance of IgA for the prevention of infection relative to that provided by serum antibody. While nasal IgA antibody was correlated ($P = 0.01$) with an increase in protection against infection, high titers of IgGa isotype antibody also appear to be associated with reducing the chance of infection in horses that were challenged. What table 4-7 does not show is the role of IgA in reducing the amount of viral shedding, however. Figure 4-13 shows the relationship between the amount of virus being shed at the peak day of shedding and nasal IgA antibody formation. An inverse correlation ($r = -0.647$) ($P = 0.009$) was seen between nasal IgA formation and virus shedding. These data suggest

that nasal IgA, present at the time of challenge infection, was associated with a reduction in the amount of virus replication during the peak period of shedding.

Table 4-4. Anti-influenza HI (HI titers), SRH (zone of hemolysis), IgGa (ELISA index), IgA (ELISA index), and CMI (stimulation index) values are from vaccinates, infected horses, and non-vaccinated negative control on day 56 (day of challenge). Clinical scores and virus shedding data are totals for the 10-day sample period following challenge. Clinical scores were based on criteria described in Table 4-1. Virus shedding is reported in $\log_{10}$ EID_{50}/mL.

Animal ID	HI	SRH	IgGa	IgA	CMI	Clinical Score	Virus Shedding
131 IM	0	0	6	35	1	5	7.2
134 IM	0	0	6	21	1.2	8	13.5
135 IM	0	0	7	29	1.1	8	6
133 IN	0	0	6	39	1.3	13	10
136 IN	0	0	6	19	1.3	14	21
137 IN	0	0	6	20	1.3	7	5
146 MLV	16	88	30	25	1.4	1	13
148 MLV	0	0	10	68	1.2	15	7
151 MLV	8	72	38	90	1.2	0	0.7
147 INCT	256	72	100	45	1.1	NC	NC
149 INCT	16	97	32	31	1.4	1	5
150 INCT	64	147	60	18	1.3	0	0
114 EIV	512	158	36	67	1.6	0	0
119 EIV	128	106	22	67	1.6	0	0
120 EIV	256	136	49	84	1	0	0
Neg Cont	0	0	8	16	1	13	12

Table 4-5. Summary table of antibody increase and presence of clinical disease and infection in vaccinated, infected, and non-vaccinated control horses after challenge. A clinical score of 3 or more was indicated by yes in the clinical disease category. The yes/no infected response was based on the presence of viral shedding after challenge.

Animal ID	Clinical Disease	Infected	HI Increase	SRH Increase	IgG Increase	IgA Increase
131 IM	YES	YES	YES	YES	YES	YES
134 IM	YES	YES	YES	YES	YES	YES
135 IM	YES	YES	YES	YES	YES	YES
133 IN	YES	YES	YES	YES	YES	YES
136 IN	YES	YES	YES	YES	YES	YES
137 IN	YES	YES	YES	YES	YES	YES
146 MLV	NO	YES	YES	YES	YES	YES
148 MLV	YES	YES	YES	YES	YES	YES
151 MLV	NO	YES	YES	NO	NO	NO
147 INCT	NC	NC	NC	NC	NC	NC
149 INCT	NO	YES	YES	YES	YES	YES
150 INCT	NO	NO	NO	YES	NO	YES
114 EIV	NO	NO	NO	NO	NO	NO
119 EIV	NO	NO	NO	NO	NO	NO
120 EIV	NO	NO	NO	NO	NO	NO
Neg Cont	YES	YES	YES	YES	YES	YES

Table 4-6. Relationship between antibody formation and clinical disease in horses at the time of challenge infection. Data were collected from horses that had received a recombinant (n = 6), MLV (n = 3), or inactivated (n = 2) vaccine, or had been previously infected (n = 3) with EIV. (Non-vaccinated control, n = 1). Proportions were determined by Fisher exact, 2-tailed analysis. A yes or no value for IgGa and IgA was based on an ELISA index value above or equal to 10 and 40 (reported in Table 4-4), respectively.

Assay Method	Antibody Formation	Horses with Clinical Disease n	(%)	P value
HI	Yes (≥ 8)	0/7	(0)	0.0001
	No	8/8	(100)	
SRH	Yes (≥ 72 mm^2)	0/7	(0)	0.0001
	No	8/8	(100)	
ELISA IgGa	Yes	0/7	(0)	0.0001
	No	8/8	(100)	
Nasal IgA	Yes	1/5	(20)	0.11
	No	7/10	(70)	

Table 4-7. Relationships between antibody formation and virus shedding in horses at the time of challenge infection. Data were collected from horses that had received a recombinant (n = 6), MLV (n = 3), or inactivated (n = 2) vaccine, or had been previously infected (n = 3) with EIV. (Non-vaccinated control, n = 1). Proportions were determined by Fisher exact, 2-tailed analysis. A yes or no value for IgGa and IgA was based on an ELISA index value above or equal to 10 and 40 (reported in Table 4-4), respectively.

Assay Method	Antibody Formation	Horses with Virus Shedding n	(%)	P value
HI	Yes (≥ 8)	3/7	(43)	0.02
	No	8/8	(100)	
SRH	Yes (≥ 72 mm^2)	3/7	(43)	0.02
	No	8/8	(100)	
ELISA IgGa	Yes	0/5	(0)	0.0005
	No	9/9	(100)	
Nasal IgA	Yes	1/5	(20)	0.01
	No	9/10	(90)	

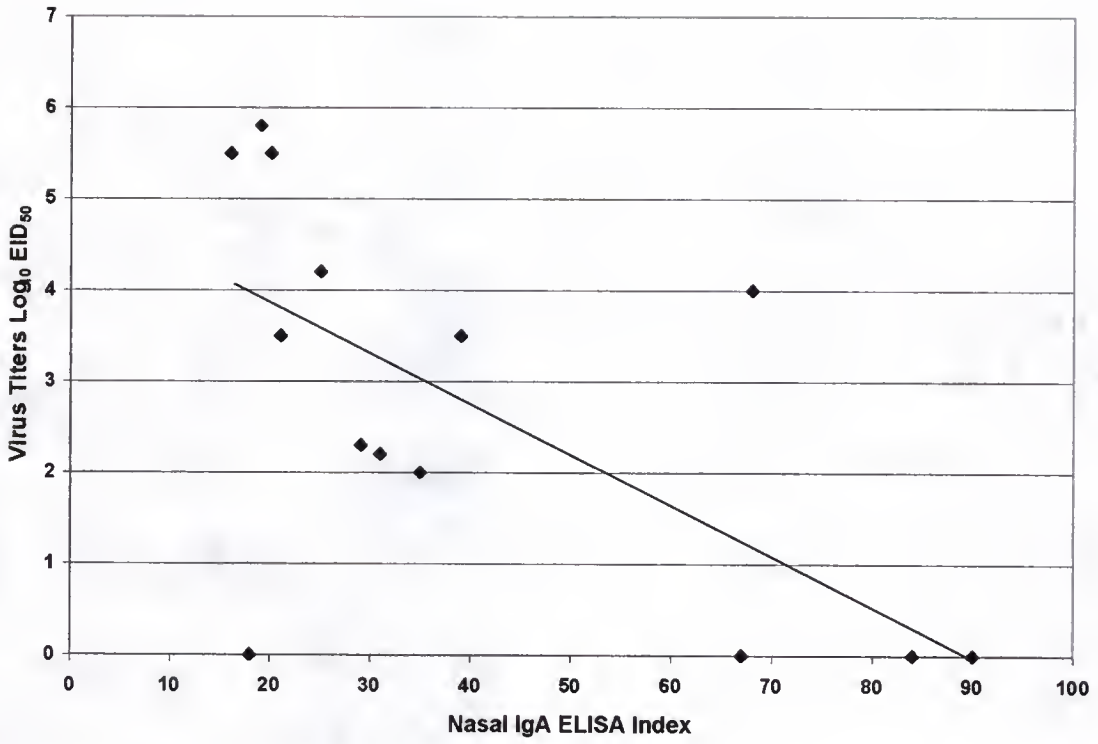


Figure 4-13. Relationship between nasal IgA antibody levels and virus shedding. IgA antibody levels were detected in horses that had received a recombinant (n = 6), MLV (n = 3), or inactivated (n = 2) vaccine, or had been previously infected (n = 3) with EIV. (Non-vaccinated control, n = 1). Nasal IgA levels were determined on the day of challenge. Virus titers are reported for the day of peak virus shedding (day 3 after challenge). A linear correlation is seen between nasal IgA formation and a reduction in viral shedding (correlation coefficient (r) = -0.6474). P = 0.009

Cytokine Determination

A summary of cytokine mRNA expression of IL-2, INF-γ, IL-4, and IL-6 for each group is displayed in figures 4-14 through 4-18. Figures 4-14, 15, 17, and 18 represent data from Realtime PCR assays following a 72-hr in-vitro stimulation of PBMC. Figure 4-16 shows the INF-γ response after an 8-hr in-vitro stimulation. As previously noted, mRNA expression of different cytokines can vary with culture conditions such as incubation time. An overall decrease in IL-2, IL-4, and IL-6 mRNA expression was noted between day zero and seven (Figures 4-14, 17, and 18). A decline was also noted on day seven after second vaccination and challenge-infection as well.

Levels of IFN-γ increased in the MLV, inactivated vaccine, and EIV-infection groups seven days after the initial vaccination/exposure on day zero. The most dramatic increase in IFN-γ production was seen after challenge in horses receiving the MLV vaccine (Figure 4-16). A In contrast, those horses receiving the inactivated-virus vaccine had only a slight increase in INF-γ production following infection. Figure 4-13 shows a reduction in IL-4 expression in all groups between PID zero and seven. In the inactivated vaccine group, a rebound of IL-4 expression occurs seven days after receiving the second vaccination inoculation. As might be expected, IL-4 levels dropped in horses vaccinated with the MLV vaccine or naturally infected. No clear pattern of IL-6 expression is indicated by the data presented here. This was suspected to be a result of culture conditions during the in-vitro stimulation prior to the PCR assay.

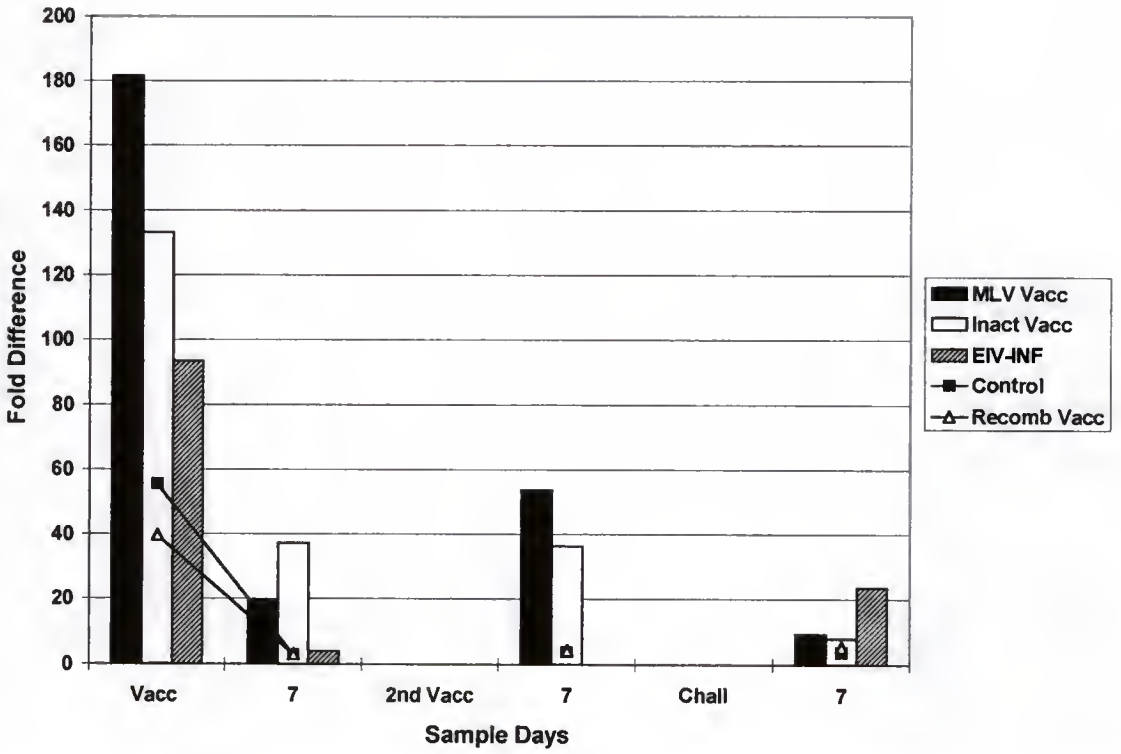


Figure 4-14. IL-2 mRNA expression in in-vivo primed peripheral blood mononuclear cells from vaccinated and infected horses during a 72-hr in-vitro stimulation with heat-inactivated equine influenza virus antigen. Equine peripheral blood mononuclear cells were stimulated in-vitro, total RNA was isolated, and cDNA was generated by reverse transcription. A Realtime PCR assay was conducted to show changes in mRNA cytokine expression at various times (day of vaccination [Vacc], 7 days after vaccination, or challenge [Chall]) as indicated. Cytokine detection assays were not done on 2nd Vacc or Chall days. Changes are expressed as an x-fold difference above a Con A control. (Vaccines and EIV-INF groups n = 3; Control n = 1)

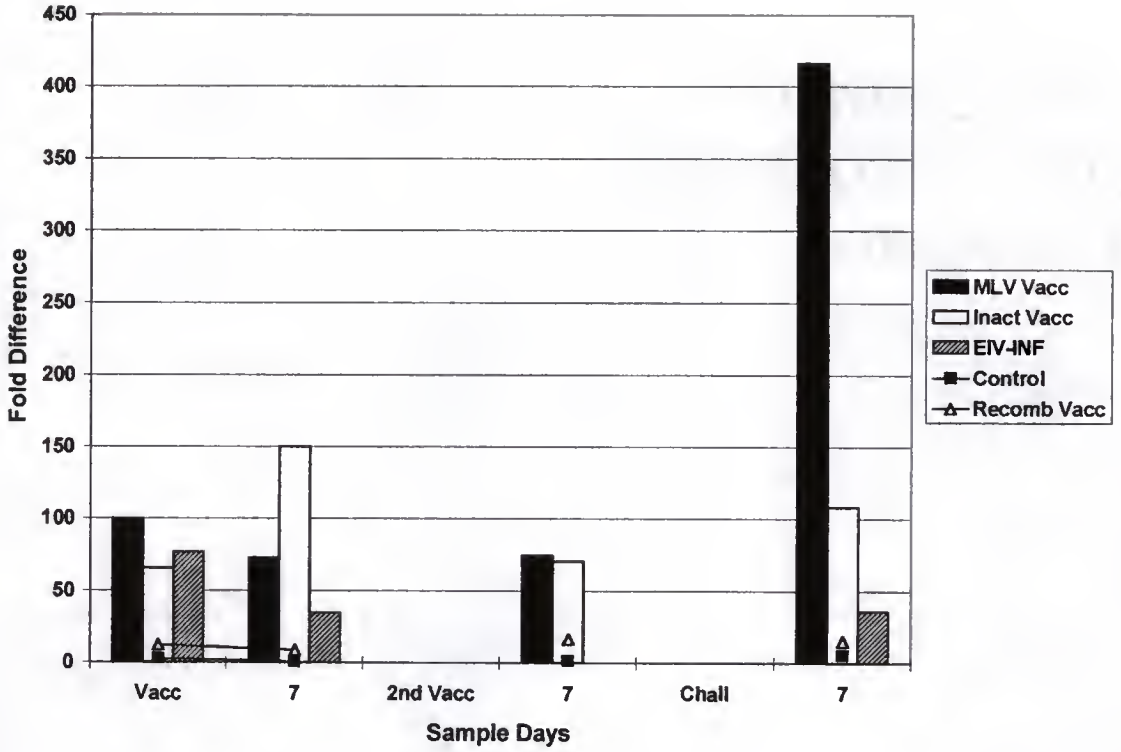


Figure 4-15. INF-γ mRNA expression in in-vivo primed peripheral blood mononuclear cells from vaccinated and infected horses during a **72-hr** in-vitro stimulation with heat-inactivated equine influenza virus antigen. Equine peripheral blood mononuclear cells were stimulated in-vitro, total RNA was isolated, and cDNA was generated by reverse transcription. A Realtime PCR assay was conducted to show changes in mRNA cytokine expression at various times (day of vaccination [Vacc], 7 days after vaccination, or challenge [Chall]) as indicated. Cytokine detection assays were not done on 2nd Vacc or Chall days. Changes are expressed as an x-fold difference above a Con A control. (Vaccines and EIV-INF groups n = 3; Control n = 1)

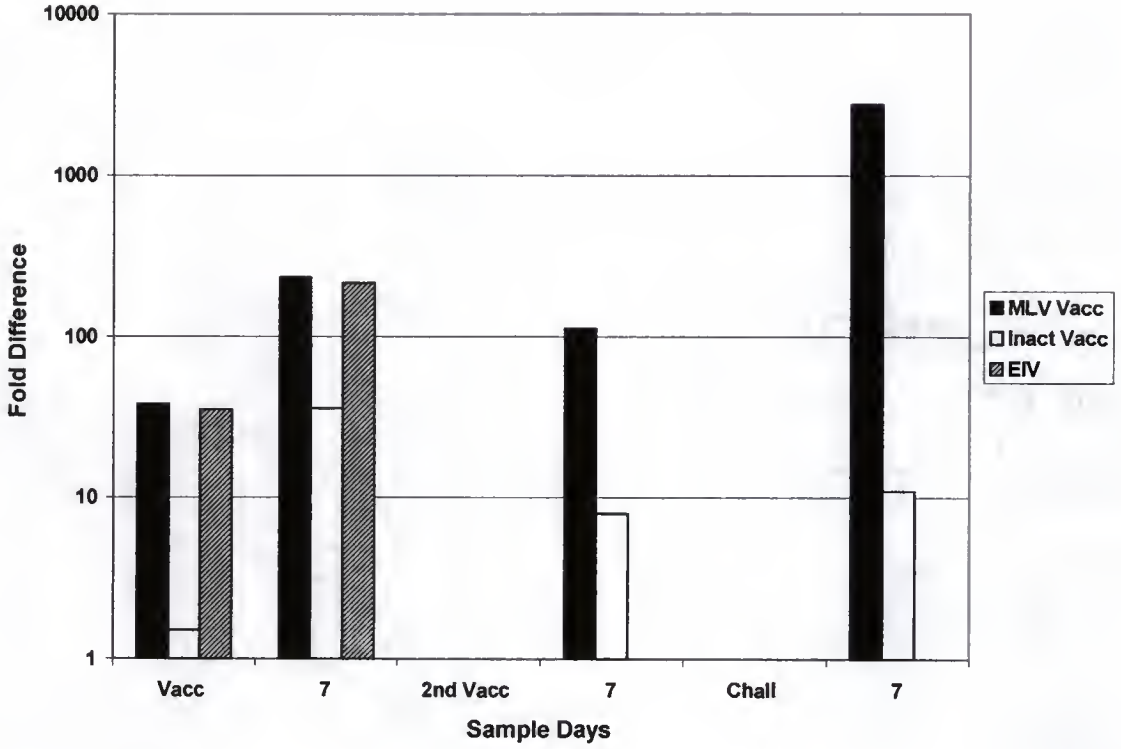


Figure 4-16. INF-γ mRNA expression in in-vivo primed peripheral blood mononuclear cells from vaccinated and infected horses during a **8-hr** in-vitro stimulation with heat-inactivated equine influenza virus antigen. Equine peripheral blood mononuclear cells were stimulated in-vitro, total RNA was isolated, and cDNA was generated by reverse transcription. A Realtime PCR assay was conducted to show changes in mRNA cytokine expression at various times (day of vaccination [Vacc], 7 days after vaccination, or challenge [Chall]) as indicated. Cytokine detection assays were not done on 2nd Vacc or Chall days. Changes are expressed as an x-fold difference above a Con A control. (Vaccines and EIV-INF groups n = 3; Control n = 1)

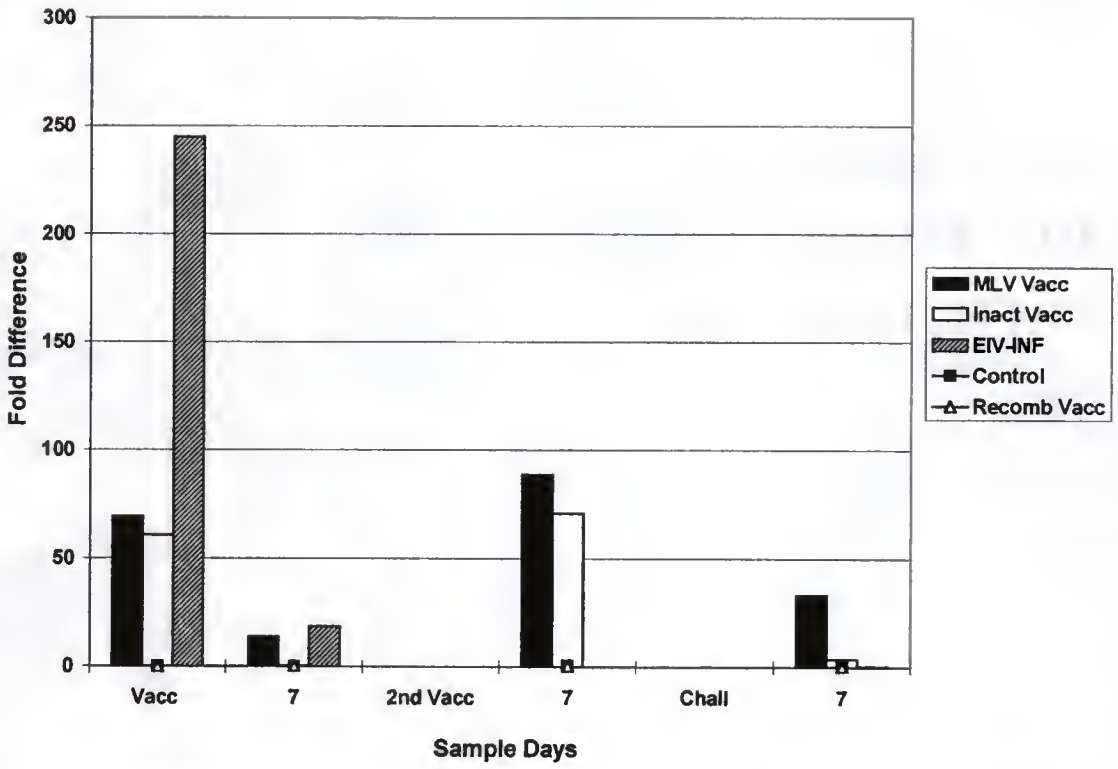


Figure 4-17. IL-4 mRNA expression in in-vivo primed peripheral blood mononuclear cells from vaccinated and infected horses during a 72-hr in-vitro stimulation with heat-inactivated equine influenza virus antigen. Equine peripheral blood mononuclear cells were stimulated in-vitro, total RNA was isolated, and cDNA was generated by reverse transcription. A Realtime PCR assay was conducted to show changes in mRNA cytokine expression at various times (day of vaccination [Vacc], 7 days after vaccination, or challenge [Chall]) as indicated. Cytokine detection assays were not done on 2nd Vacc or Chall days. Changes are expressed as an x-fold difference above a Con A control. (Vaccines and EIV-INF groups n = 3; Control n = 1)

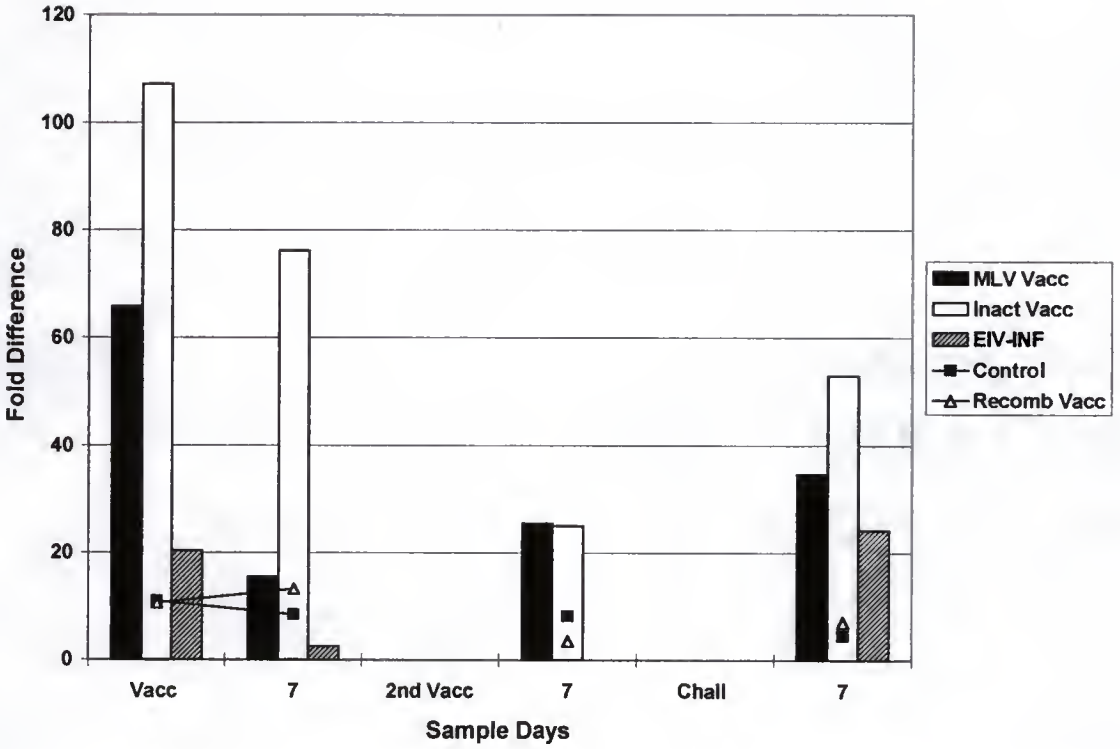


Figure 4-18. IL-6 mRNA expression in in-vivo primed peripheral blood mononuclear cells from vaccinated and infected horses during a 72-hr in-vitro stimulation with heat-inactivated equine influenza virus antigen. Equine peripheral blood mononuclear cells were stimulated in-vitro, total RNA was isolated, and cDNA was generated by reverse transcription. A Realtime PCR assay was conducted to show changes in mRNA cytokine expression at various times (day of vaccination [Vacc], 7 days after vaccination, or challenge [Chall]) as indicated. Cytokine detection assays were not done on 2nd Vacc or Chall days. Changes are expressed as an x-fold difference above a Con A control. (Vaccines and EIV-INF groups n = 3; Control n = 1).

Discussion

In this study, we hypothesized that vaccination of horses with a novel recombinant DNA vaccine would result in protection against infection and clinical disease following influenza challenge. The response was to be compared to that seen after immunization with commercially available vaccines and experimental infection with EIV. Multiple analytical methods were used to confirm the systemic and local antibody response, and CMI after vaccination and subsequent challenge-infection. Furthermore, viral shedding and clinical signs were assessed to determine the level of protection provided by each form of immunization in horses.

The recombinant DNA vaccine used in this study was a type III vectored vaccine. Type III vectored vaccines basically consist of a non-pathogenic or gene-deleted organism into which specific genetic material is inserted for the purpose of stimulating an immune response. This method of vaccination addresses some of the concerns with reversion of virulence seen in live-attenuated virus vaccines. In the present study, a recombinant EHV-4 that had been designed to contain gene deletions (gE and US2) within the US region was used. The genes for HA and NA from a strain of EIV (A/equine/2/Kentucky/94) were inserted in the US2 region. Based on virus isolation and serology (data not shown), it appears that the EHV-4 vector used in the present study did not replicate in the horse. This was confirmed despite verification of viral RNA expression by rt-PCR and protein expression by Western blot analysis of virus grown in Vero cells. Two possible explanations for the failure of the virus to replicate within the horse are: 1) the EHV-4 vector lost the ability to replicate due to over attenuation, or 2) the presence of pre-existing antibodies to field-strains of EHV-4 or EHV-1. Based on the epidemiology of infection with equine herpes viruses, it would seem reasonable that a robust EHV-4 vector would grow in horses. The first of the two explanations is supported by other reports regarding over-attenuation due to gene deletions associated with replication within the host animal. In one study, the inability of a laboratory strain of EHV-4 virus to grow in horses was attributed to the deletion of gE and gI genes (Matsumura, Kondo et al., 1998). This was confirmed in two other studies

where repeated passage of the same strain resulted in the spontaneous loss of several genes, two of which encoded gE and gI. In pigs, glycoproteins gI and gp63 of pseudorabies virus are homologs of gE and gI, respectively, and play a role in the invasion and spread of virus in the nervous system of pigs (Kritas, Pensaert et al., 1994). In other reports, the gE gene deletion was attributed to the loss of virulence in bovine (Sussman, Maes et al., 1995) and felines (van Engelenburg, Kaashoek et al., 1994). The vector used in our experiments contained a functional gI gene, but had the thymidine kinase and gE genes deleted. The most significant correlation between this study and others is: 1) the similar gE gene deletion in the EHV-4 vector and 2) the inability of the vector to replicate in horses despite their lack of pre-existing antibodies.

The MLV vaccine used in this study was generated from a temperature sensitive reassortant virus that has been investigated for efficacy against challenge with live virus in horses (Holmes, Lamb, Coggins, & et.al, 1991). In the present study, the use of a cold-adapted MLV vaccine resulted in systemic and local antibody formation and a cell-mediated component of the immune response without indications of clinical signs. Further, the use of MLV vaccines has been shown in humans (Boyce, Gruber, Sannells, & et.al, 2000) (Tomoda, Morita, Kurashige, & Maassab, 1995) and horses (Lunn, Hussey, Sebing, Rushlow, Radecki, Whitaker-Dowling, Youngner, Chambers, Holland, Jr., & Horohov, 2001) to generate an immune response similar to those described here. Cold-adapted, attenuated influenza vaccines are produced by serial passage at low temperature with a current field strain. Reassortants are selected that contain the six internal genes (conferring attenuation and cold-adaptation) of the donor strain (Maassab, 1969) and HA and NA from the current strain. The loss of the non-virulent phenotype is a source of concern when using MLV vaccines. Other published work has shown that MLV vaccines are safe and effective against EIV infection (Mumford, Jessett et al., 1999). In these studies, immunization using a MLV vaccine was not associated with clinical disease typically seen after infection with wild-type virus.

The method of experimental challenge (instilled or nebulized virus) and the strain of EIV have been shown to affect the level of circulating antibodies in horses (Mumford, 1999a). These reports have relevance to future studies that should investigate better methods to administer intranasal vaccines. The present study concluded that the inoculation device provided by the manufacturer of the MLV vaccine could be improved. The MLV vaccine was instilled using a 15-cm long tube. The manufacturer's instructions for inoculation were followed and resulted in a loss of vaccine from the nostril. It seems reasonable that an applicator that "mists" the vaccine deep within the nostril would improve the inoculum-delivery process. Furthermore, because nebulized virus is believed to more closely mimic natural infection, a study to determine immunity and protection following aerosolized inoculation of a cold-adapted vaccine seems warranted.

An antibody response to EIV was induced by the inactivated vaccine after two inoculations and was further increased after infection. However, like past studies, the current data show a rapid decline in antibody titers. The duration of this study precluded the evaluation of antibody past 14 days after challenge-infection. Protection against clinical disease can be obtained from infection-induced immunity in horses. However, the partial protection provided by inactivated-virus vaccines can actually perpetuate a level of endemic infection among vaccinates and susceptible horses alike. Therefore, repeated vaccinations are required to maintain protective levels of antibody for an extended duration (Mumford, Wood, Folkers, & Schild, 1988;Mumford, Jessett, Dunleavy, Wood, Hannant, Sundquist, & Cook, 1994a; Mumford, Jessett, Rollinson, Hannant, & Draper, 1994b;Wood, Mumford, Folkers, Scott, & Schild, 1983). The present study supports these previous data by showing a rapid decline in serum antibody levels after vaccination. The current study also demonstrated the need for at least two inoculations with an inactivated-virus vaccine to induce serum antibody titers equal to those needed for protection against severe clinical disease.

Horses within this study were infected in a manner that provided a consistent challenge-dose between subjects. Several studies are summarized in a report showing a range of antibody titers associated with protection based on challenge strain, dose, and the method of inoculation (Mumford, 1991). Likewise, the level of clinical disease resulting from experimental infection depends on the infectious dose (un-published data), the strain used (Powell, 1991), and method of delivery (Mumford, 1991;Mumford, Hannant, & Jessett, 1990; Wood, Mumford, Folkers, Scott, & Schild, 1983). In the present study, a heterologous challenge-strain of EIV (A/equine/2/Kentucky/95) was used in horses immunized with a recombinant DNA, an inactivated-virus, or a MLV vaccine containing antigen from A/equine/2/Kentucky/94, 92, and 91, respectively. Due to the similar antigenic properties of these 4 A/equine/2/Kentucky strains, an immune response providing protection against severe clinical disease was likely. The ability of a vaccine to eliminate viral shedding is dependent upon the degree of antigenic relatedness between the vaccine and challenge strain (Daly, 1996). While vaccine-efficacy studies typically incorporate a homologous challenge-virus, outbreaks among horses and humans are often associated with field-strains that are not contained in vaccines routinely used. However, there are reports that address heterotypic immunity in horses induced by various vaccines as well. In a recent safety and efficacy study, a MLV vaccine containing the A/equine/2/Kentucky/91 strain of EIV was used to demonstrate a heterotypic immune response after challenge with A/equine/2/Kentucky/98 and A/equine/2/Saskatoon (Holland, Chambers, Townsend, Cook, Bogdan, & Lunn, 1999). Furthermore, conventional inactivated virus vaccines have been shown to induce antibodies to antigenically related strains (Fort Dodge Commercial Information., 1998).

Not surprising, the recombinant vaccinates lacking an antibody and cell-mediated response, shed virus and had clinical scores similar to those of the unvaccinated control. Both the inactivated and MLV vaccines resulted in a reduction of virus shedding and clinical scores over that seen in the unvaccinated control. However, complete protection against infection was not achieved by the use of the MLV vaccine. Based on previous reports regarding the efficacy of

MLV vaccines, a reduction in virus shedding and clinical disease was expected (Boyce, Gruber, Sannells, & et.al, 2000;Holland, Chambers, Townsend, Cook, Bogdan, & Lunn, 1999;Tomoda, Morita, Kurashige, & Maassab, 1995). Increases in nasal IgA antibody (Boyce, Gruber, Sannells, & et.al, 2000;Tomoda, Morita, Kurashige, & Maassab, 1995), IFN-γ, IL-2, lymphocyte proliferation (Tomoda, Morita, Kurashige, & Maassab, 1995), and systemic antibody (Lunn, Hussey, Sebing, Rushlow, Radecki, Whitaker-Dowling, Youngner, Chambers, Holland, Jr., & Horohov, 2001) have been reported following immunization with MLV vaccines. Theoretically, the comprehensive immune response induced by MLV vaccines should provide greater protection against infection and clinical disease, when compared to an inactivated-virus vaccine. In the present study, the number of test animals, a low response by one horse (# 148 in the MLV vaccine group), and the un-related death of another (# 147 in the inactivated vaccine group) prior to challenge may have skewed virus shedding and clinical score data. Single radial hemolysis, HI, and ELISA data confirmed the poor response by horse number 148 in the MLV group. This horse shed higher titers of virus for more days than others in either commercial vaccine group. In addition, the loss of horse number 147 in the inactivated vaccine group, reduced the number of challenged vaccinates within that group to two animals. It is feasible that these events effected the distribution of virus shedding and clinical score data. To address confounding factors such as non or low responders and the loss of test subjects, enrollment of a greater number of horses in each group would have been statistically more appropriate.

Horses initially infected with EIV were completely protected against challenge as demonstrated by the absence of viral shedding and clinical disease. Horses within this group served as a positive control for protection against clinical disease. The effectiveness of natural infection to generate a comprehensive immune response leading to complete protection has been reported elsewhere and was demonstrated in the present study. After the initial infection, horses shed virus at titers near that typically seen during natural infection (Mumford, Jessett, Rollinson, Hannant, & Draper, 1994b). However, an important point to consider is that conventional

methods to isolate and measure virus include mucous secretions from the nostril of horses. While this may not necessarily correlate with the actual amount of virus shed during natural infection, consistency in sampling methods among published reports allow comparisons to be made.

In this study, the pattern of increases in HI antibodies was similar to those detected by SRH and ELISA methods. An increase in cross-reacting antibodies to EIV that were detected by the HI and SRH assays is consistent with previous data provided by the manufactures of the inactivated and MLV vaccines. Mean HI titers of 1:170 reported for horses in the EIV-infection group and 1:64 in one inactivated-virus vaccinate (# 150) correlated with complete protection against infection. Furthermore, a second horse receiving the inactivated-virus vaccine had HI titers of 1:16 and reduced clinical signs as compared to the unvaccinated control.

Depending upon the method of experimental infection and the strain used, SRH titers ranging from 65 to 154 mm^2 are generally considered adequate to provide protection against infection (Mumford, 1999b). In another study, an inactivated vaccine containing carbomer adjuvant rather than aluminium phosphate was shown to induce SRH levels greater than or equal to 154 mm^2 and 85 mm^2 that provided protection against infection and severe clinical disease, respectively (Mumford, Wilson, Hannant, & Jessett, 1994). In the present study, SRH titers induced by the inactivated-virus vaccine, averaged from 96 to 146 mm^2 on the day of challenge-infection and resulted in the reduction of severe clinical disease and infection respectively. The mean SRH antibody titer in horses receiving the inactivated-virus vaccine was higher (not significantly higher) than those receiving the MLV vaccine (mean SRH titer of 53 mm^2) on the day of challenge. Horses experimentally infected with live virus were completely protected (mean SRH titer of 133 mm^2) against challenge infection. In summary, results from this study demonstrate a range of vaccine-induced HI and SRH antibody titers. However, a correlation between HI and SRH antibody levels and protection against infection and disease is not always straightforward.

ELISA data showed a similar pattern of antibody production to that detected by both HI and SRH. In the present study, IN and IM vaccination with the recombinant DNA vaccine failed to induce an antibody response. The failure of the vaccine to induce EIV-specific antibody is speculated to be associated with the recombinant vector and not the site of inoculation, however. Not withstanding, IN inoculation of a viable recombinant DNA vaccine against EIV may have promising results regarding the induction of a comprehensive immune response. Depending upon the location of inoculation and type of vaccine used, IgGa, IgGb, IgGc, or IgG(T) production can be differentially induced (Lunn, Soboll, Schram, Quass, McGregor, Drape, Macklin, McCabe, Swain, & Olsen, 1999) (Nelson, Schram, McGregor, Sheoran, Olsen, & Lunn, 1998). Horses in the recombinant vaccine group and the negative control showed comparable increases 14 days after challenge. Furthermore, the pattern and level of antibody production in the recombinant (following first and second vaccination) and control animals were similar to the EIV-infection group following their initial exposure. Thus, the data for the recombinant vaccinates reflect a primary response by naïve animals, indicating the failure of the recombinant vaccine to induce antibody formation.

While "poor responders" have been described previously (Mumford, 1999a), horses vaccinated with the recombinant vaccine were not likely candidates based on their immune response after challenge-infection. Inactivated-virus and MLV vaccines and experimental infection induced EIV-specific antibodies in most of the horses used in this study. One horse, number 148, showed an initial response following first vaccination. However, using serum antibody formation as an indicator, this horse responded rather weakly upon second vaccination and did not increase again, subsequent to infection. This horse developed severe clinical disease upon challenge. Reasons for poor or non-responders are unclear and due to the variability they contribute to a group response, the number of experimental animals used in a vaccine study should account for this type of confounding data. As noted before, the weak antibody response

and subsequent clinical disease may have contributed to the apparent poor performance of the MLV vaccine compared to that of the inactivated-virus vaccine.

Few significant differences in the serum anti-influenza antibody titers were noted between the inactivated-virus and MLV vaccinates. Several significant ($p<0.05$) increases were noted in each on various days when compared to baseline values recorded on PID 0. Horses receiving the inactivated or MLV vaccines did not show increases in serum antibodies until 14 days after the second vaccination (day of challenge). However, vaccination with the inactivated or the MLV vaccine primed horses for a more rapid increase in serum antibodies after challenge-infection than those receiving the recombinant vaccine or unvaccinated control. Unfortunately, the duration of the study precluded continued monitoring for antibodies and their relative decline in each group. The pattern of serum antibody detected in the present study was similar to that seen in mice when they were either vaccinated with an inactivated vaccine or experimentally infected with influenza virus (A/PR/8/34. H1N1) (Matsuo, Iwasaki, Asanuma, Yoshikawa, Chen, Tsujimoto, Kurata, & Tamura, 2000). In the Matsuo report, the IgG response was strongest in the infection group and was correlated to Th1 cytokine increases.

Consistent with other reports regarding the duration of antibodies after vaccination and infection (Mumford, 1998), the present study suggests a requirement for repeat immunization due to a rapid decline (in weeks). Horses in the EIV-infection group had a decrease in antibody titers starting at six weeks after experimental infection and even sooner in the vaccine-induced horses. Furthermore, the MLV and inactivated vaccine groups had only a minimal response until a second inoculation was administered. The decline in antibody titers to a level that provides protection against severe disease but possibly results in sub-clinical infection, has been suggested as a cause for the endemic nature of equine influenza (Wilson, 1993). A rapid decline in antibody titers after vaccination was seen in the current study. Similar to other reports (Hannant, Mumford et al., 1988) (Townsend, Morely, et al. 1999), horses in this study that had low (less than or equal to 1:64) serum anti-influenza antibody titers at the time of challenge infection, subsequently

seroconverted (Tables 4-4 and 4-5). However, those with high serum antibody titers did not have an increase in anti-influenza antibody. Seroconversion in those horses lacking antibody is consistent with the current paradigm that anti-influenza antibody production requires viral replication. Thus, those horses with high serum anti-influenza antibody effectively reduced the availability of influenza antigen required for serum antibody production. Collectively, the serology data in tables 4-6 and 4-7 would indicate that serum antibody formation has an important role in protection against clinical disease. These data are consistent with other reports that have shown serum antibody is associated more with protection against severe clinical disease and less with preventing infection (Mumford, 1999; Hannant, Easeman, and Mumford, 1999). Thus, this study supports the idea of repeated vaccinations to maintain protection against infection or reduce the severity of clinical disease.

While several studies have characterized the systemic antibody response to vaccination against influenza in horses, there are fewer reports describing mucosal IgA production (Lunn, Olsen, Soboll, McGregor, Drape, Macklin, McCabe, & Swain, 1999; Lunn, Hussey, Sebing, Rushlow, Radecki, Whitaker-Dowling, Youngner, Chambers, Holland, Jr., & Horohov, 2001;Nelson, Schram, McGregor, Sheoran, Olsen, & Lunn, 1998; Hannant, Easeman, & Mumford, 1999) . In the present study, the local IgA antibody response to vaccination and infection was detected by sampling secretions of the upper respiratory tract. Similar to the serum IgGa antibody response, mucosal IgA production was not detected in horses after vaccination with the recombinant vaccine. As might be expected, significant ($P<0.01$) increases in IgA over background levels were detected in the MLV vaccinates on PID 56 (day of challenge) with a subsequent increase after challenge. Likewise, the local IgA response to aerosolized virus in the EIV-infection group was significant two weeks after inoculation. These data are consistent with other reports indicating a priming effect from the use of MLV cold-adapted vaccines (Boyce, Gruber, Sannells, & et.al, 2000). Horses appeared to benefit from the MLV vaccine because they responded with an increase in IgA titers quicker than un-vaccinated horses. Nasal IgA increases

in the inactivated-virus vaccinates lagged behind all other groups after challenge infection. One possible explanation may involve the cytokine-induced Th-type response in the inactivated-virus vaccinates. Others have shown that, in mice, vaccination with inactivated influenza virus will induce a Th-2 type response corresponding to increases in IL-4 (Matsuo, Iwasaki, Asanuma, Yoshikawa, Chen, Tsujimoto, Kurata, & Tamura, 2000). Interleukin-4 up-regulation will promote a Th2 type response resulting in serum IgG production and suppression of IFN-γ expression (Roitt, 1997). In a previous report, INF-γ mRNA expression correlated with IgA production during natural infection in mice or a MLV vaccine used in children (Matsuo, Iwasaki, Asanuma, Yoshikawa, Chen, Tsujimoto, Kurata, & Tamura, 2000;Tomoda, Morita, Kurashige, & Maassab, 1995).

In the current study, horses previously infected and, to a lesser degree, those immunized with the MLV vaccine, were protected against infection and severe clinical disease. Secretory IgA is the principal mediator to prevent infection with intracellular pathogens (Mestecky & McGhee, 1987) and its intracellular transport has been demonstrated in foals (Galan, Timoney et al., 1986) and mice (Renegar & Small, Jr., 1991). Furthermore, the importance of IgA during vaccination and infection with influenza in mice (Renegar & Small, Jr., 1991;Tamura, Funato, Hirabayashi, Kikuta, Suzuki, Nagamine, Aizawa, Nakagawa, & Kurata, 1990) and humans (Boyce, Gruber, Sannells, & et.al, 2000;Clements, Betts, Tierney, & Murphy, 1986;Small, 1990;Tomoda, Morita, Kurashige, & Maassab, 1995) has been described.

Local immunity consists of IgA effectively neutralizing viruses within the epithelial cell as well as the lumenal surface (Mazanec, Coudret et al., 1995b). During influenza infection, newly synthesized viral HA and NA move from the trans-golgi network to the lumenal surface of epithelial cells via endosomal compartments containing IgA. This has been demonstrated experimentally by infection of Madin Darby canine kidney cells (MDCK) with influenza or Sendai virus (Mazanec, Coudret, & Fletcher, 1995a). A key membrane protein

directly related to the transport of IgA from the basolateral to the apical surface is the polymeric immunoglobulin receptor (pIgR). This protein, along with other unique binding activities associated with IgA, is a key feature in the mechanisms of the mucosal antibody response in mammals (Mazanec, Nedrud, Kaetzel, & Lamm, 1993). An 80-kDa protein originally designated "the secretory piece" and now referred to as the secretory component (SC) is also involved in the movement of pIgA across the epithelial cell of mucosal associated tissues. The SC is a proteolytic product of an intracellular protein (pIgR) responsible for the recognition, binding, and transport of IgA from the basolateral to the apical membrane of epithelial cells.

The recombinant DNA vaccine failed to induce both serum and local antibodies. Further, the antibody response after challenge was not significantly different from that seen in the unvaccinated control. There appeared to be a differential antibody response resulting from vaccination with either the inactivated-virus or MLV vaccine. Vaccination with the inactivated-virus vaccine resulted in significant increases in serum IgGa but not nasal IgA antibody by the 14th day after a second immunization. In contrast, MLV vaccinates had a significant rise in nasal IgA but not serum IgGa antibodies on the 14th day after receiving a second inoculation. The serum IgGa and nasal IgA response of horses in the EIV-infection group was not significantly different on the day of challenge than that seen in horses receiving the inactivated-virus and MLV vaccines respectively.

Consistent with other reports (Asanuma, Koide, et al. 1995; Hannant, Easeman, et al. 1999), anti-influenza IgA appeared to play a primary role in significantly preventing viral shedding in horses within the present study (Figure 4-13). The present study also suggests that horses with high anti-influenza serum antibody titers are provided with an increase in the level of protection against infection as well (Table 4-7). Additional testing of nasal secretions for mucosal IgG antibody may provide further evidence of role of systemic antibody during

challenge. In other animals, virus neutralizing IgA antibody has been reported after infection with influenza and has been proposed as a possible mechanism for homotypic and heterotypic protection {Asanuma, Koide, et al. 1995 793 /id}. Here, we describe reduced viral shedding in horses with nasal antibody formation against a heterotypic challenge strain of A/equine/2/ Kentucky EIV.

Thus, this study suggests that: 1) the recombinant DNA vaccine failed to elicit a systemic or local antibody response, 2) a strong serum and local EIV-specific antibody response similar to that resulting from natural infection was induced by repeated immunization with an inactivated-virus and MLV vaccine, respectively, 3) a second inoculation with the MLV vaccine was required to induce a significant local IgA response, 4) repeated vaccinations are required to maintain antibody levels considered protective against infection and severe clinical disease, and 5) serum and local antibody formation are important in providing protection against clinical disease and virus shedding, respectively.

This study demonstrated a weak antigen-specific lymphoproliferative response in vitro, generated by in-vivo primed PBMC from horses following vaccination against or infection with EIV. The ability of lymphocytes to respond to a stimulus in vitro demonstrates a functional capacity and is applicable to clinical immunology. The lymphoproliferative response can be used to measure not only an individuals basic functional ability but can also be applied to qualify previous exposure to infectious disease, cancer, malnutrition, stress, and other environmental factors (Fletcher, Klimas, Morgan, & Gjerset, 1992). At the most basic description, the proliferation assay measures the number of generated and surviving cells in culture after the addition of a stimulus.

Similar to the serology data, horses receiving the recombinant vaccine did not have a measurable lymphocyte proliferative response after in-vitro stimulation with EIV antigen until 14 days after challenge. Studies have shown that recombinant DNA vaccines delivering HA and NP from influenza virus can induce a cell-mediated response in mice (Bender, Ulmer et al., 1998).

There are few reports demonstrating the induction of cell mediated immunity in horses using recombinant DNA technology, however. In the present study, the lack of a cell-mediated response in the recombinant vaccinates was most likely attributed to an over-attenuated vector and vaccine failure. Possibly, due to the inability of the vector virus (EHV-4) to replicate in the horse, a cell-mediated immune response was not induced. No previous studies have investigated an antigen-specific proliferation response to vaccination against EIV using a MLV vaccine. In the present study, horses immunized with the MLV had an increased proliferation response over baseline values following a second vaccination. The MLV vaccinates had a further increase after challenge infection as well. As might be expected, horses receiving the inactivated-virus vaccine did not show an increase in the lymphocyte proliferation response until after challenge-infection. Increases in the cell-mediated arm of the immune response in mice have been reported by vaccination with MLV (Tomoda, Morita, Kurashige, & Maassab, 1995) but not inactivated-virus (Matsuo, Iwasaki, Asanuma, Yoshikawa, Chen, Tsujimoto, Kurata, & Tamura, 2000) vaccines. For vaccines to be effective in preventing infection and reducing the severity of disease, they must induce humoral, local, and cell-mediated immunity. While the MLV did not completely abrogate viral shedding, and antibody titers were low following vaccination, the severity of clinical disease was reduced over that seen in the unvaccinated control. These data, along with those collected from horses receiving the inactivated vaccine show that a correlation between high antibody titers or CMI and the protection provided by each upon challenge-infection, is not always straight forward. Increases in the stimulation indices were moderate but consistent with other reports (Ellis, Bogdan, & Kanara, 1995) (Hannant, 1994) (Kruse, Moriabadi, Toyka, & Rieckmann, 2001). In 2 studies, one with horses (Hannant, 1994), and the other in humans (Kruse, Moriabadi, Toyka, & Rieckmann, 2001), vaccinates had been immunized more than twice and/or had previous exposure to virus. Additionally, another study reported on the proliferative response to vaccination against equine herpes virus (Ellis, Bogdan, & Kanara, 1995). Despite the difference in the vaccination schedule reported in these and the present study,

increases in the stimulation indices were all within a three-fold difference of base-line values. The level of in-vitro proliferation that constitutes a biologically significant response is unknown. However, it is important to note that horses within the present study had no prior exposure to influenza and thus the relative increase in the stimulation index most likely reflected an antigen-specific response.

In the present study, PBMC isolated from whole blood were assayed to investigate in-vivo priming through vaccination and infection. Considering that influenza is a mucosal-associated virus, lymphocytes within the pool of circulating PBMC that are primed to an antigen-specific response are probably fewer in number than could be found in respiratory-associated lymph tissue. The ability to detect an intranasal-vaccine-induced immune response in horses may have been improved by obtaining pulmonary lymph node associated lymphocytes. Lymphoid cell aggregates in nasal-associated lymphoid tissue (NALT), have been recognized as a mucosal-associated lymph tissue that provide a source of antiviral IgA antibody-forming-cells (Asanuma, Inaba et al., 1995;Kuper, Koornstra et al., 1992). Furthermore, lymphocytes from NALT in mice were shown to express increased IFN-γ mRNA (Matsuo, Iwasaki, Asanuma, Yoshikawa, Chen, Tsujimoto, Kurata, & Tamura, 2000), an indicator of cell-mediated immunity. While the fate of memory lymphocytes is still controversial, it is believed that upon antigen stimulation, they home to the site of exposure and local lymph nodes (Sprent & Tough, 1994). The homing of primed lymphocytes to pulmonary-associated lymph tissue would increase the density of responder memory cells over that seen in the peripheral blood; thus increasing the sensitivity of an in-vitro proliferation assay system.

Reagents to investigate cytokine production in horses are limited. This, therefore, precluded our ability to assay culture supernatants or nasal secretions for cytokine protein-expression in the present study. An alternative approach utilizes the vesicular stomatitis virus (VSV) plaque reduction assay to detect INF-γ in culture supernatants (Yilma, McGuire et al.,

1982). The VSV plaque reduction assay has been used to measure INF-γ increases in the supernatants of equine PBMC after vaccination against EHV-1 (Ellis, Steeves, Wright, Bogdan, Davis, Kanara, & Haines, 1997). The characterization and generation of monoclonal antibodies to equine cytokines has been limited (Horohov, 1999;Lunn, Sobol, Swiderski, Horohov, & Olsen, 1999) and ELISA techniques to measure cytokine production in horses has not been described. However, published studies have reported on the development and future application of these reagents (Lunn, Sobol, Swiderski, Horohov, & Olsen, 1999). The generation of primers to detect equine cytokine mRNA expression in a PCR assay has been described (Giguere & Prescott, 1998). Furthermore, primers for equine cytokine mRNA have been constructed for use in "Realtime" PCR assays (Giguere & Prescott, 1999). In the same study, Giguere reports on the increased sensitivity of Realtime methods over that of conventional PCR. Others have previously demonstrated the specificity and high sensitivity of real-time PCR to detect mRNA expression from a limited amount of starting material (Gibson, Heid et al., 1996). Here, Realtime PCR was used to determine changes in cytokine mRNA expression following in-vitro stimulation of in-vivo primed PBMC.

mRNA cytokine expression can vary depending on in-vivo priming and antigen presentation during in-vitro stimulation. Whereas cell-mediated immune effector cells recognize epitopes that are conserved among all types of A strain influenza, antibodies, and to a certain extent Th cells, recognize epitopes on antigenic-drift-variants from different subtypes (Bastin, Rothbard et al., 1987).

Helper T lymphocytes appear to play a key role in directing the Th1 or Th2 response to viral infection through the production of cytokines (Guidotti & Chisari, 2000). While INF-γ can initiate a direct antiviral state in concert with tumor necrosis factor-α (TNF-α), other cytokines, such as IL-2, IL-4, and IL-6, have a indirect effect through paracrine and autocrine upregulation (Thomson, 1998). The antiviral activity of cytokines led to the discovery of INF-γ and its role in

the antiviral state of un-infected cells (Staeheli, 1990). Furthermore, INF-γ enhances the production of MHC class I but not II molecules in T cells, induces Th1 cells to produce both IL-2 and IL-2 receptors (IL-2R), activates macrophages to phagocytose and destroy microorganisms, and promotes antibody-dependent, cell-mediated cytotoxicity (ADCC) (Tizard, 1996). Consistent with a Th1 type response, INF-γ has been reported to increase after infection with influenza in mice (Matsuo, Iwasaki, Asanuma, Yoshikawa, Chen, Tsujimoto, Kurata, & Tamura, 2000).

In the present study, IL-2 cytokine expression in response to the recombinant vaccine was similar to that of the un-vaccinated control. Based on the serological and CMI data, it was not surprising that the horses receiving the recombinant vaccine did not show changes in IL-2, INF-γ, IL-4, or IL-6 until after infection. The effectiveness of DNA vaccines leading to cytokine-secreting lymphocytes has been described (Huygen, Content et al., 1996; Xiang, Spitalnik et al., 1995). Other studies have described a cellular immune response following immunization with an NP-expressing recombinant DNA vaccine against human influenza (Ulmer, Fu et al., 1998) (Fu, Friedman et al., 1997). In the Ulmer study, lymphocyte proliferation and cytokine production was induced by inoculation with a DNA vaccine. This study characterized the role of $CD4^+$ cells in a Th1 type response resulting in the production of IL-2 and INF-γ. Thus, one might speculate that a cytokine-controlled response to recombinant DNA vaccination against EIV is feasible. What is not known is the possible differential cytokine increase related to the specific antigen-protein (HA, NA, and NP in the case of influenza vaccines) expressed by the vector. A previous study showed that a DNA NP-expressing vaccine induced a strong MHC class I-restricted CTL response resulting from the secretion of Th1-type cytokines (Ulmer, Donnelly et al., 1993). It also concluded that the heterosubtypic immunity was not due to antibody. Therefore, a study to determine the Th-type response and subsequent release of cytokines following immunization with a recombinant DNA vaccine expressing either NP, or HA and NA warrants further investigation.

Horses immunized with the MLV vaccine showed a relative increase in IL-2 and INF-γ mRNA expression seven days after the first and second vaccination and again after challenge. Consistent with a Th1 type response, IL-2 and INF-γ increases have been reported in mice at seven days after influenza infection (Matsuo, Iwasaki, Asanuma, Yoshikawa, Chen, Tsujimoto, Kurata, & Tamura, 2000). Similar cytokine responses were seen in humans after immunization with a cold-adapted, MLV vaccine against influenza (Tomoda, Morita, Kurashige, & Maassab, 1995). Seven days after the second vaccination, IL-4 expression had decreased in the MLV vaccinates. In contrast, horses receiving the inactivated-virus vaccine showed a relative decrease in IL-2 and INF-γ and an increase in IL-4 and IL-6 expression. Similar data has been reported in mice where the use of an inactivated vaccine resulted in lower levels of IL-2 and INF-γ compared to those seen after infection (Matsuo, Iwasaki, Asanuma, Yoshikawa, Chen, Tsujimoto, Kurata, & Tamura, 2000). While there is evidence that Th1 cytokines like INF-γ generate a suppressive effect on Th2-type cells, IL-4 can, in turn, inhibit the production and function of Th1 cells (Mosmann & Coffman, 1989). Other studies have shown that the treatment of mice with IL-4 was associated with the inhibition of antiviral protection mediated by CTL activity (Sharma, Ramsay et al., 1996). Changes in cytokine expression in the present study were similar to that seen in other published data in that a cytokine-induced Th1 or Th2-like response emerged depending on the type of vaccine used or natural infection. Horses in the MLV vaccine and EIV-infection groups had a predominantly Th1-like cytokine response. In contrast, those in the inactivated-virus vaccine group showed Th-2-like cytokine expression. This may explain the differential pattern of local and systemic antibody production seen in the MLV and inactivated-virus vaccinates, respectively. Horses with a Th-1 response (MLV vaccinates) had higher levels of mucosal IgA antibody and those with a Th-2 response (inactivated-virus vaccinates) had a strong serum IgGa antibody response. In mice increases in IgA and IgG during infection or vaccination against influenza are associated with INF-γ and IL-4 expression, respectively. In addition to INF-

γ, IL-2 has been shown to increase after influenza infection in mice (Matsuo, Iwasaki, Asanuma, Yoshikawa, Chen, Tsujimoto, Kurata, & Tamura, 2000). In the same study, where mice were infected with influenza, IL-6 expression was equally strong and postulated as participating in a Th0 type response correlating with IgA production.

Interleukin-6 expression was uniformly lower than other cytokines in the vaccinated and infected horses. However, there was a moderate increase in IL-6 in the EIV-infection group. There is evidence that the so-called "early" cytokines, produced by non-immune cells, are the cause of several clinical signs associated with influenza (Van Reeth, 2000). The early cytokines, such as IFN-α, TNF-α, and IL-1, are found at the site of influenza infection (lung lavage samples) (Skoner, Gentile et al., 1999). They are followed by IL-6 and other chemotactic cytokines that are associated with local inflammation, fever, lethargy, and anorexia (Bielefeldt-Ohmann, 1995). Systemic levels of IL-6 are typically lower or un-detectable indicating a higher local production. In humans, IL-6 peaks at two to three days after inoculation with influenza and correlates with nasal titers of virus and clinical signs (Hayden, Fritz et al., 1998). Blood samples were collected on the 7th day following vaccination or infection possibly explaining the relatively low expression levels in each group.

In summary, the lymphocyte proliferation data, even though subtle, was similar to serology and virus isolation data in regards to the failure of the recombinant vaccine to induce an EIV-specific response. In contrast, the MLV vaccine induced a moderate cell-mediated response in vaccinates prior to and again after challenge. In contrast, a lymphocyte proliferation response was not generated after vaccination with an inactivated-virus vaccine. The relative increases in cytokine mRNA expression in horses in this study followed a Th1-like response in the MLV vaccinates and EIV-infection group and a Th2-like response in the inactivated-virus vaccine.

Future Studies

The results from this study were similar to previous investigations regarding the efficacy of MLV and inactivated vaccines against severe clinical disease or infection with EIV. In contrast, the use of a recombinant DNA vaccine, consisting of an equine herpes virus vector with a deletion in the gE gene region, failed to replicate and induce an immune response. Despite the failure of this particular recombinant vector, deficiencies in other vaccine preparations support the need for continued development of recombinant DNA vaccines. Recombinant DNA vaccines expressing combinations of NP, HA, and NA genes are certainly within the realm of current technology. Therefore, mechanisms of cell-mediated immunity including CTL activity and cytokine production should be investigated to better characterize the systemic and local antibody response after vaccination. To further investigate and confirm the reasons for vaccine failure, organ and tissue culture techniques would be a comparatively inexpensive approach. Using both the original and a re-designed construct with the gE gene intact, the EHV-4 vector should be grown in primary equine respiratory tract cells. These culture conditions, unlike that using Vero cells, more closely mimic the host and provide a better chance to estimate the viability of the vector in vivo. Yet another approach which most closely mimics the animal model, would be the use of equine tracheal-ring cultures to confirm growth of the recombinant vector.

Modified-live vaccines present a viable alternative to inactivated-virus preparations and further development of products that provide complete protection to current circulating strains of EIV are necessary. A recent study investigated the safety of a MLV vaccine that had been made commercially available within the last year (Lunn, Hussey, Sebing, Rushlow, Radecki, Whitaker-Dowling, Youngner, Chambers, Holland, Jr., & Horohov, 2001). This MLV vaccine was the same one used in the present study. Unlike the method used in the present study, Lunn et al

administered the vaccine by nebulization. During such a study, the potential benefits of nebulisation versus instillation may include an increase in efficacy. Because antigen preparation and the location of delivery have been shown to effect the immunological response, studies addressing these variables are needed. These studies should include, but not be limited to, MLV and recombinant DNA vaccines. Furthermore, the same study should delineate the differential cytokine expression that may exist due to changes in location and method of inoculation. Based on the outcome of such an experiment, it seems reasonable that an inoculation device that "mists" rather than "instills" the vaccine onto the mucosal airway surface could be developed.

When horses were immunized with the inactivated vaccine, they formed a significant serum antibody response. In contrast, the inactivated vaccine did not induce a local antibody response. The opposite pattern of antibody formation was noted when horses were immunized with the MLV vaccine. The MLV vaccine induced a significant local but weak serum antibody response. By the 14th day after receiving a second inoculation of either the MLV or the inactivated vaccine, horses were immune to severe clinical disease when challenged. Based on these observations, a study that investigates the possible advantages of alternating MLV and inactivated-virus vaccination over that seen by the use of either one alone might have merit.

The ability to design vaccines that provide complete protection against infectious pathogens is somewhat limited by our ability to characterize the effector mechanism that control the immune response. Specifically, cytokines have been shown to play a key role in the type (Th-1 or 2) responses induced by vaccination. Recent developments in equine-specific reagents that allow the further characterization of the complex interactions of the equine immune system are promising. Even though the number of reports regarding cytokines in horses is small compared to that in humans or mice, the importance of this field of research has been recognized. Recent and ongoing studies will hopefully provide the necessary equine-specific reagents to develop better vaccines against EIV.

CHAPTER 5

GENERAL SUMMARY

The purpose of this study was to investigate the immune response of horses to immunization with a recombinant DNA vaccine against equine influenza. The study furthermore compared the efficacy of the recombinant vaccine to that of a modified-live and inactivated-virus vaccine. A "positive control" for a comprehensive immune response was accomplished by infecting a third group of horses with live equine influenza virus.

Gold-standard techniques and optimized variations of others provided the methodology to measure the immune response of horses. Standardized HI and SRH assays were useful in assessing serum antibody formation. An ELISA technique was shown to possess sensitivity enabling the detection of seroconversion at an earlier time point than either HI or SRH. Furthermore, a successful model for infection with aerosolized EIV at an EID_{50} of 10^8 was established within our laboratory and provided a good test of various vaccines used in the animal study. The use of rayon-tipped swabs was shown to be an efficient method to obtain nasal secretions for IgA analysis. Based on the blastogenic response to mitogen and EIV antigen and the expression of cytokine mRNA, this study showed that peripheral blood mononuclear cells collected from horses could be cryopreserved, thawed, and used to assess in-vivo priming by in-vitro techniques. Here, a 4-dy proliferation assay consisting of in-vitro stimulation with heat-inactivated EIV was capable of detecting an antigen-specific response. Furthermore, Realtime PCR proved to be a sensitive and specific assay for cytokine mRNA expression in equine PBMC.

The overall results of the animal study indicate that the recombinant DNA vaccine failed to induce an immune response prior to challenge and did not prime horses for an anamnestic

response. Based on information collected here and previous work, it is presumed that the EVH-4 vector did not replicate in the horse. By comparison, the inactivated and MLV vaccine provided protection against severe clinical disease. Protection against severe clinical disease in the inactivated-virus vaccinates was associated with serum antibody formation. In contrast, the MLV vaccinates had a significant local immune response that provided some level of protection upon challenge. The relative increase in immunity within vaccinates was as expected when compared to that seen in the positive (EIV-infection group) and negative (non-vaccinated) controls. Horses initially infected with EIV showed both a systemic and local antibody response but only a moderate cell-mediated response. This resulted in complete protection from infection upon challenge. The non-vaccinated control remained seronegative until after challenge, by which time viral shedding and clinical disease were confirmed. In all groups, protection against clinical disease was primarily mediated by serum antibody while nasal IgA antibody showed a greater associated with protection against infection. This study further showed that at least two immunizations are required to induce a significant immune response for naïve horses and suggests the need for repeated vaccination to maintain protection against infection with EIV.

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BIOGRAPHICAL SKETCH

I was born in Tampa, Florida on April 14, 1960. Both parents, Boyce and Marceil Sweat are native Floridian and reside in Seffner, Florida. I attended kindergarten through the 12th grade at Temple Heights Christian Schools in Tampa. The school was associated with Temple Heights Baptist Church where I spent much of my extra time. In high school, I participated in football, baseball, wrestling, and was a member of the concert and jazz band. From the time I was 12 years of age, my father and I enjoyed many hours on the backs of horses exploring the, at that time, un-developed areas of northeast Tampa.

After high school, I attended Hillsborough Community College and then the University of South Florida (USF) in Tampa. During that time, I was employed by veterinary clinics with intentions of becoming a veterinarian. After graduation from USF, I married my wife, Susan, and went to work for the Harbor Branch Oceanographic Institute in Ft. Pierce, Florida.

Upon my return to the Tampa-Bay area in 1989, I started a 10-year employment with the Florida Department of Natural Resources (presently Florida Fish and Wildlife Conservation Commission) at the Florida Marine Research Institute in St. Petersburg, Florida. During that time, I was involved in various aspects of manatee conservation management and research. In 1992, I helped establish the Marine Mammal Pathobiology Laboratory (MMPL) which had a primary focus of determining causes of mortality in the Florida manatee. In 1996, I received a Master's in Microbiology from USF. While helping host the 46th Annual Conference on Wildlife Disease, I made contacts that eventually led to the graduate research program here at the University of Florida. Upon completing my graduate studies, I look forward to spending more

time with my wife and daughter Jessica, as well as returning to my hobbies that include saltwater fishing, and riding my horse and my Harley Davidson.

REV. 02-01

THE GRADUATE SCHOOL - UNIVERSITY OF FLORIDA

REPORT ON THESIS OR DISSERTATION AND/OR FINAL EXAMINATION

To the Dean of the Graduate School: Date: June 8, 2001

Mr. James Mark Sweat Social Security No. 263-79-9362

has submitted, in partial fulfillment of the requirements for the degree of

Doctor of Philosophy (PHD) in the College of VETERINARY MEDICINE (VM)

a Dissertation entitled:

The Immune Response in Horses to Vaccination Against EquineInfluenza Virus. A Comparison of DNA Recombinant, Inactivated-virus, and Modified-live virus Vaccines.

This Dissertation has been examined by all members of the candidate's supervisory committee and has been

✓ APPROVED _____ REJECTED _____ NOT APPLICABLE

The committee has examined the candidate on June 8, 2001 (date) in accordance with the regulations governing the Final Examination and has adjudged his/her performance

✓ SATISFACTORY _____ UNSATISFACTORY

Exceptions or qualifications are noted as follows:

Signatures of members of Supervisory Committee*: Names of faculty representatives attending final examination:*

Name		Name	Field
[signature] CHAIR	266-87-2492 SSN	PAUL GIBBS	VIROLOGY
[signature]	214-50-8509 SSN	BRADLEY S BENDER, MD	MEDICINE
[signature]	268 28 4063 SSN	Parker A. Smith Jr MD	Immunology
[signature]	522 49 6543 SSN	Jorge A Hernandez	Epidemiology
[signature]	423-96-7734 SSN	Calvin M. Johnson	Vet. Med.
[signature] External Member (Dissertation Only)	391-94-6755 SSN	D. PAUL LUNN	U. WISCONSIN-MADISON

Recommended by: [signature] Department Chair

Approved by: [signature] Dean Of The College

DIRECTIONS: The original copy of this form is signed by all members of the supervisory committee and by the Dean of the College. Send the original copy to the Graduate School Editorial Office if thesis or dissertation or to the Graduate School Records Office if final exam.

*All members of the Supervisory Committee, including the external member, must be members of the Graduate Faculty and must be present for the final doctoral or master's examination and sign this form.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Paul Gibbs, Chair
Professor of Veterinary Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Bradley Bender,
Professor of Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Jorge Hernandez,
Associate Professor of Epidemiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Calvin Johnson,
Professor of Veterinary Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Paul Lunn,
Associate Professor and Associate Dean
of Clinical Affairs

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Parker Small,
Professor of Medicine

This dissertation was submitted to the Graduate Faculty of the College of Veterinary Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 2001

C. H. Courtney for

Joseph DiPietro, Dean
College of Veterinary Medicine

Winfred Phillips, Dean
Graduate School